

Hydrogen Peroxide Generation by the Pepper Extracellular Peroxidase CaPO2 Activates Local and Systemic Cell Death and Defense Response to Bacterial Pathogens^{1[W][OA]}

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Reactive oxygen species (ROS) are responsible for mediating cellular defense responses in plants. Controversy has existed over the origin of ROS in plant defense. We have isolated a novel extracellular peroxidase gene, *CaPO2*, from pepper (*Capsicum annuum*). Local or systemic expression of *CaPO2* is induced in pepper by avirulent *Xanthomonas campestris* pv *vesicatoria* (*Xcv*) infection. We examined the function of the *CaPO2* gene in plant defense using the virus-induced gene silencing technique and gain-of-function transgenic plants. *CaPO2*-silenced pepper plants were highly susceptible to *Xcv* infection. Virus-induced gene silencing of the *CaPO2* gene also compromised hydrogen peroxide (H_2O_2) accumulation and hypersensitive cell death in leaves, both locally and systemically, during avirulent *Xcv* infection. In contrast, overexpression of *CaPO2* in *Arabidopsis* (*Arabidopsis thaliana*) conferred enhanced disease resistance accompanied by cell death, H_2O_2 accumulation, and *PR* gene induction. In *CaPO2*-overexpression *Arabidopsis* leaves infected by *Pseudomonas syringae* pv *tomato*, H_2O_2 generation was sensitive to potassium cyanide (a peroxidase inhibitor) but insensitive to diphenylene iodonium (an NADPH oxidase inhibitor), suggesting that H_2O_2 generation depends on peroxidase in *Arabidopsis*. Together, these results indicate that the *CaPO2* peroxidase is involved in ROS generation, both locally and systemically, to activate cell death and *PR* gene induction during the defense response to pathogen invasion.

Upon recognition of pathogens, plants activate a battery of defense responses, including the oxidative burst, the hypersensitive response (HR), cell wall fortification, and defense-related protein synthesis (Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997; Jones and Dangl, 2006). One of the most rapid defense reactions to pathogen attack is the oxidative burst, which leads to the transient production of large amounts of reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$; Mehdy, 1994; Lamb and Dixon,

1997; Kawano, 2003; Laloi et al., 2004). The oxidative burst occurs during the HR in plants following the perception of pathogen avirulence signals (Hammond-Kosack and Jones, 1996; Lamb and Dixon, 1997). The rapid generation of O_2^- or its dismutation product H_2O_2 at the onset of the HR has been documented in many plant-pathogen interactions (Doke, 1983; Grant et al., 2000). ROS produced in the oxidative burst not only protect against invading pathogens but also function as signaling molecules to activate plant defense responses (Tenhaken et al., 1995; Jabs, 1999). During the defense response in plant cells, ROS can inhibit pathogens by strengthening host cell walls via the oxidative cross-linking of glycoproteins, such as the Pro-rich protein (Bradley et al., 1992), or by directly killing pathogens (Levine et al., 1994).

Several enzymes have been implicated in the generation of ROS during defense responses in a number of plant-pathogen interactions. An NADPH-dependent oxidase system was initially found in mammalian neutrophils (Babior, 1984; Apel and Hirt, 2004). NADPH oxidase, known as the respiratory burst oxidase, catalyzes the production of O_2^- by the one-electron reduction of molecular oxygen using NADPH as an electron donor. The rapid transformation of O_2^- into H_2O_2 during plant-pathogen interactions was first observed in potato (*Solanum tuberosum*) tubers infected with an avirulent race of *Phytophthora infestans* (Doke, 1983; Park and Doke, 2005). *Rboh* (respiratory burst oxidase homologs) genes encoding gp91^{phox} (the catalytic subunit of the NADPH oxidase of phagocytes) have been

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found in rice (*Oryza sativa*; Groom et al., 1996), *Nicotiana benthamiana* (Yoshioka et al., 2003), and *Arabidopsis* (*Arabidopsis thaliana*; Desikan et al., 1998; Keller et al., 1998; Torres et al., 1998; Laloi et al., 2004; Torres and Dangl, 2005). Interestingly, plant NADPH oxidases have an additional N-terminal calcium-binding EF hand, which is absent from the phagocyte NADPH oxidases, suggesting direct activation by Ca^{2+} ions in plant cells in contrast to mammalian cells (Keller et al., 1998). Knockout mutations of *Atrboh* genes eliminate ROS production in the defense response of *Arabidopsis* to avirulent pathogens (Torres et al., 2002). Pathogen-induced, NADPH oxidase-derived ROS were recently demonstrated to play a role in suppressing the spread of cell death in *Arabidopsis* (Torres et al., 2005).

In addition to plant NADPH oxidases, peroxidases have been proposed as alternative producers of ROS (Apel and Hirt, 2004; Bindschedler et al., 2006). Peroxidases catalyze the oxidoreduction of various substrates using H_2O_2 . Many peroxidases localize to vascular and apoplastic compartments. Notably, peroxidases in the cell wall can generate apoplastic H_2O_2 at neutral to basic pH in the presence of reductants in plant cells (Bolwell et al., 1998). In French bean (*Phaseolus vulgaris*) suspension-cultured cells treated with a cell wall elicitor from *Colletotrichum lindemuthianum*, cell wall peroxidase rather than NADPH oxidase has been proposed to be the major source of ROS (Bolwell and Wojtaszek, 1997; Bolwell et al., 1998, 1999). In this system, elicitors are recognized by putative receptors located on the plasma membrane to activate ion channels in plant cells. The movement of H^+ and other ions such as Ca^{2+} , K^+ , and Cl^- may contribute to a transient alkalization of the extracellular matrix, ultimately activating the pH-dependent cell wall peroxidase to generate ROS (Zimmerlin et al., 1994).

ROS from the oxidative burst were proposed to orchestrate the plant defense response and hypersensitive cell death during incompatible plant-pathogen interactions (Levine et al., 1994). Since then, there has been increasing support for a key role of ROS as triggers of cell death. Recently, transgenic plants with perturbed levels of cellular antioxidants have demonstrated the important role of ROS in different plant cell death events (Lorrain et al., 2003). *Arabidopsis* mutants of the *atrbohD* and *atrbohF* genes exhibit reduced hypersensitive cell death and an absence of ROS in response to avirulent *Pseudomonas syringae* (Torres et al., 2002). Similarly, *Nbrboh*-silenced *N. benthamiana* plants not only cannot accumulate H_2O_2 but also are compromised for HR activation following *P. infestans* infection (Yoshioka et al., 2003). Moreover, ROS have also been proposed to mediate a reiterative signal network underlying systemic acquired resistance (SAR; Alvarez et al., 1998; Lee and Hwang, 2005). The infection of lower pepper (*Capsicum annuum*) leaves with an avirulent strain of *Xanthomonas campestris* pv *vesicatoria* (*Xcv*) induces SAR in the uninoculated upper leaves (Lee and Hwang, 2005). SAR is associated with a rapid increase in the oxidative burst, with the generation of

O_2^- and the subsequent accumulation of H_2O_2 in plant tissues (Lamb and Dixon, 1997). For instance, the SAR response of pepper plants is accompanied by a systemic microoxidative burst that generates H_2O_2 and a systemic expression of defense-related genes in uninoculated leaves (Lee and Hwang, 2005). ROS induce the coordinate expression of a set of so-called SAR genes. There are indications that H_2O_2 acts as an intercellular or intracellular secondary messenger for the systemic induction of various defense genes in plants (Orozco-Cárdenas et al., 2001). More recently, NADPH oxidase has been demonstrated to mediate the rapid, systemic generation of ROS in response to virus infection in *Arabidopsis* (Love et al., 2005).

In this study, we have isolated and functionally characterized a novel extracellular peroxidase gene pepper *PEROXIDASE2* (*CaPO2*) that is implicated in disease resistance. We used the virus-induced gene silencing (VIGS) technique in pepper (Baulcombe, 1999) and ectopic expression in *Arabidopsis* (Clough and Bent, 1998) as efficient forward genetic approaches to define the functions of the *CaPO2* gene in plant defense. *CaPO2*-silenced pepper plants were highly susceptible to infection by *Xcv*. Moreover, silencing of the *CaPO2* gene not only compromised the oxidative burst and the HR in primary infected leaves (leading to a local response) but also microoxidative bursts and micro-HR in secondary leaves elsewhere in the same plant (leading to a systemic response) during infection by avirulent *Xcv*. In contrast, transgenic *Arabidopsis* plants that constitutively overexpressed the *CaPO2* gene exhibited enhanced bacterial disease resistance, as well as the oxidative burst and the HR.

RESULTS

The *CaPO2* cDNA Encodes Extracellular Peroxidase

The *CaPO2* cDNA was isolated from a cDNA library made from pepper leaves infected with the avirulent strain Bv5-4a of *Xcv* by using a macro-cDNA array method (Jung and Hwang, 2000). The *CaPO2* cDNA consists of 1,120 bp, including a 19 bp poly-A tail, and codes for a protein of 322 amino acids with a predicted molecular mass of 34.6 kD and a pI of 8.04. The translated *CaPO2* amino acid sequence was used as a query for a database search (<http://www.ncbi.nlm.nih.gov/blast/>), which revealed that it showed homology to extracellular peroxidases. The putative amino acid sequence encoded by the *CaPO2* cDNA clone was 85% identical to lignin-forming anionic peroxidase of wood tobacco (*Nicotiana glauca*; accession no. B56555) and 33% identical to *CaPO1* (accession no. AAL35364; Do et al., 2003). Computational analysis of the *CaPO2* peptide sequence using PROSITE (www.expasy.org/prosite) revealed that it possesses a proximal heme-binding ligand (H-196), a peroxidase active site signature, and four conserved disulphide bridges (Supplemental Fig. S1).

Computational analysis of the predicted protein sequence revealed that CaPO2 is likely to be a secreted protein (PSORT, 82%; TargetP, 94.8%) and that it has a signal peptide cleavage site between 27-Ala(A) and 28-Gln(Q) (Supplemental Fig. S1). To determine the subcellular localization of the CaPO2 protein, the *soluble-modified GFP* (*smGFP*) gene was fused to *CaPO2* under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 1A). The *35S:CaPO2:smGFP* fusion construct and a *35S:smGFP* control were introduced into onion (*Allium cepa*) epidermal cells by particle bombardment. As shown in Figure 1B, the CaPO2:smGFP fusion protein was localized in the extracellular matrix, while the control smGFP was uniformly distributed throughout the cell.

CaPO2 Is Strongly Induced in Leaves Infected by Avirulent *Xcv*

The expression of the pepper *CaPO2* gene in different organs was examined by RNA gel-blot analysis. As shown in Figure 2A, *CaPO2* transcripts were only faintly detected in the floral tissue of healthy pepper plants. However, they were not detected in leaves, stems, or roots, or in green or red fruits. This observation suggests that the *CaPO2* gene is not constitutively expressed in healthy plant organs, except for the flower. RNA gel-blot analysis of the *CaPO2* gene was performed to determine whether the gene is induced in pepper leaves during compatible and incompatible interactions with *Xcv* (Fig. 2B). The *CaPO2* and *CaBPR1* (pepper basic PR1 protein) genes were strongly induced in leaves inoculated with the avirulent (incompatible) strain Bv5-4a. In particular, the induction of *CaPO2* could be detected as early as 5 h after Bv5-4a infection, i.e. before the appearance of the HR. However, *CaPO2* transcripts were only faintly detected in mock-inoculated leaves (10 mM MgCl₂) or in leaves inoculated with the virulent (compatible) strain Ds1.

The same RNA samples were probed with the *CaBPR1* as a positive control. This result suggests that the *CaPO2* gene is strongly induced during the resistance response of pepper plants to *Xcv* infection.

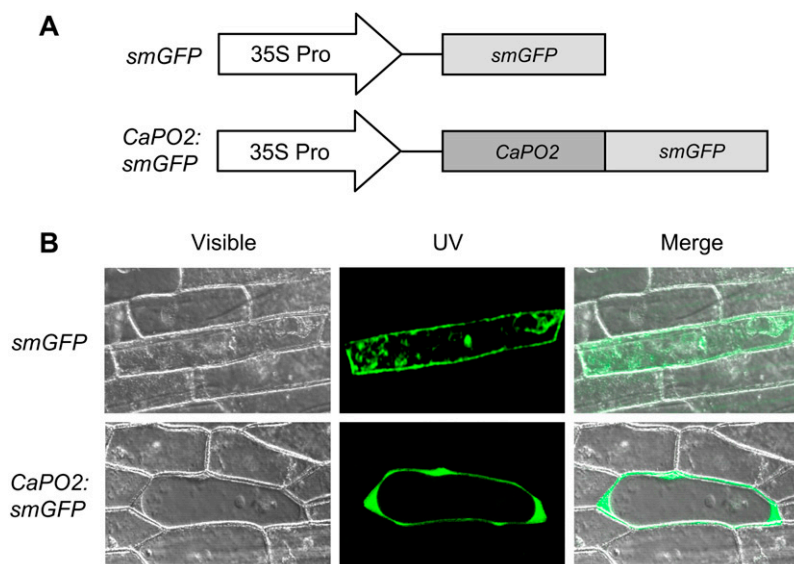
Avirulent *Xcv* Infection Induces Oxidative Bursts and the HR in Pepper Plants

To dissect the roles of extracellular CaPO2 peroxidase in H₂O₂ generation and in the HR during the defense response of pepper plants to *Xcv*, we stained leaves with diaminobenzidine (DAB; a histochemical reagent for H₂O₂) and with trypan blue to detect the micro-HR. Strong and differential staining with DAB and trypan blue was observed in both primary and secondary leaves 24 h after inoculation of primary leaves with the *Xcv* avirulent strain Bv5-4a. Intriguingly, infiltration of secondary leaves with potassium cyanide (KCN; a peroxidase inhibitor) or diphenylene iodonium chloride (DPI; an NADPH oxidase complex inhibitor) effectively inhibited the subsequent induction of the microoxidative bursts and of micro-HR in uninoculated secondary leaves (Fig. 3A). Notably, *CaPO2* transcripts in secondary leaves were also eliminated by KCN or DPI treatment, although the pepper pathogenesis-related gene (*CaBPR1*) was induced under these conditions (Fig. 3B). Thus, we hypothesize that the extracellular peroxidase CaPO2 and NADPH oxidase are both required for systemic microbursts and micro-HR in pepper leaves.

Silencing of the *CaPO2* Gene in Pepper Increases Disease Susceptibility Accompanied by Compromised Defense-Related Gene Expression

We observed that the *CaPO2* gene was strongly induced during the incompatible interaction of pepper plants with the *Xcv* avirulent strain Bv5-4a (Fig. 2B). To examine the effect of loss of function of the *CaPO2*

Figure 1. Analysis of the subcellular localization of the CaPO2 protein by transient expression of the *CaPO2:smGFP* construct in onion epidermal cells. A, Schematic of the *smGFP*-tagged *CaPO2* construct and of a control construct. The *smGFP* gene was fused to the 3' region of the *CaPO2* gene. B, Transient expression of *smGFP* or *CaPO2:smGFP* in onion epidermal cells was detected by confocal laser-scanning microscopy 24 h after biolistic transformation.



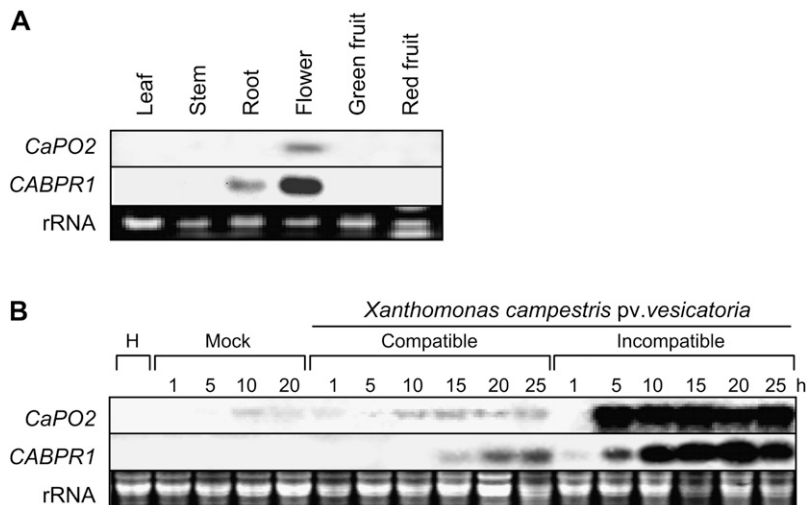


Figure 2. RNA gel-blot analyses of expression of the *CaPO2* and *CaBPR1* genes in pepper plants. The membranes were hybridized with 32 P-dCTP labeled *CaPO2* and *CaBPR1* probes. Equal loading of total RNA (20 μ g per lane) was verified by visualizing rRNA on a gel stained with ethidium bromide. A, Organ-specific expression of the *CaPO2* and *CaBPR1* genes in pepper plants. B, Expression of *CaPO2* and *CaBPR1* in pepper leaves at various time points after inoculation with the virulent strain Ds1 (compatible) and the avirulent strain Bv5-4a (incompatible) of *Xcv* at the six-leaf stage. H, Healthy leaves; Mock, leaves treated with 10 mM $MgCl_2$.

gene in the incompatible interaction, we silenced the gene in pepper plants using the tobacco rattle virus (TRV)-based VIGS technique with the full-length *CaPO2* open reading frame (Liu et al., 2002; Chung et al., 2004). To assess the efficiency of VIGS, *CaPO2* transcript levels were examined by reverse transcription (RT)-PCR in empty vector control (TRV:00) and

CaPO2-silenced (TRV:*CaPO2*) pepper leaves 24 h after inoculation with *Xcv* (Fig. 4). *CaPO2* transcripts were not detected upon virulent *Xcv* infection of the *CaPO2*-silenced leaves; however, they remained at a slightly visible level in the avirulent *Xcv*-infected leaves. To determine whether the expression of defense-related genes is affected by bacterial infection in the silenced

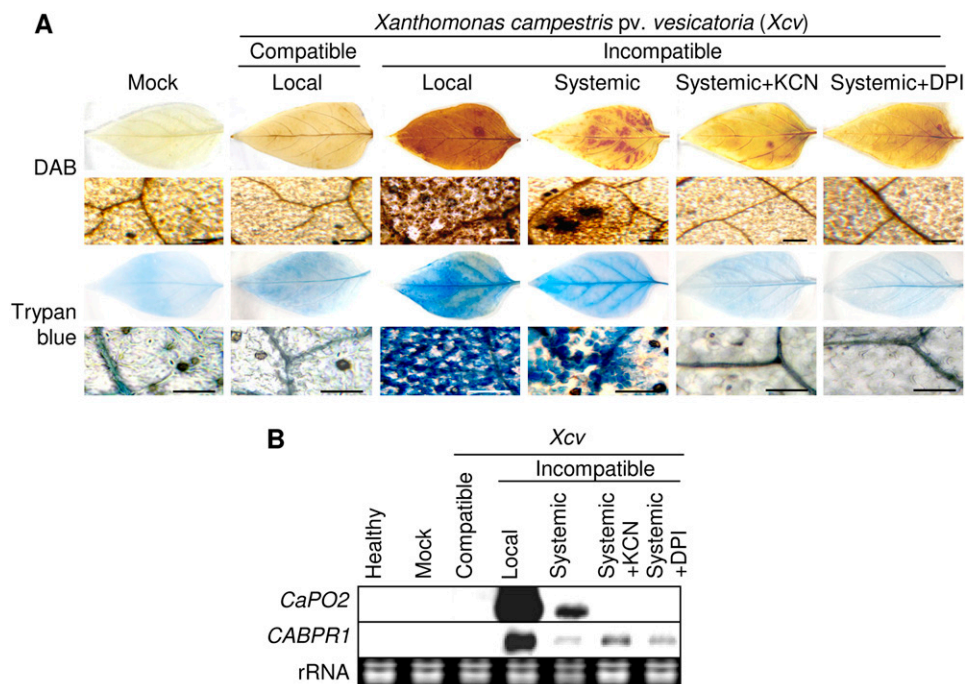


Figure 3. Peroxidase- and NADPH oxidase-dependent oxidative burst and HRs in pepper plants. A, DAB and trypan blue staining of the primary and secondary pepper leaves at different times after inoculation of primary leaves with the avirulent strain Bv5-4a (10^8 cfu mL^{-1}) of *Xcv*. Secondary leaves were also simultaneously infiltrated with either 1 mM KCN (peroxidase inhibitor) or 2.5 μ M DPI (NADPH oxidase complex inhibitor) immediately after *Xcv* inoculation. *Xcv*-infected local leaves were harvested for DAB and trypan blue staining 1 and 24 h after inoculation, respectively; however, uninoculated systemic leaves were stained with both agents 24 h after inoculation. Bars = 200 μ m. B, RNA gel-blot analysis of expression of *CaPO2* and *CaBPR1* in primary and secondary pepper leaves 24 h after inoculation with the *Xcv* avirulent strain Bv5-4a at the six-leaf stage. The membrane was successively hybridized with 32 P-dCTP-labeled *CaPO2* and *CaBPR1* probes. Local, *Xcv*-inoculated leaves; Systemic, uninoculated leaves distant from the *Xcv* infection site.

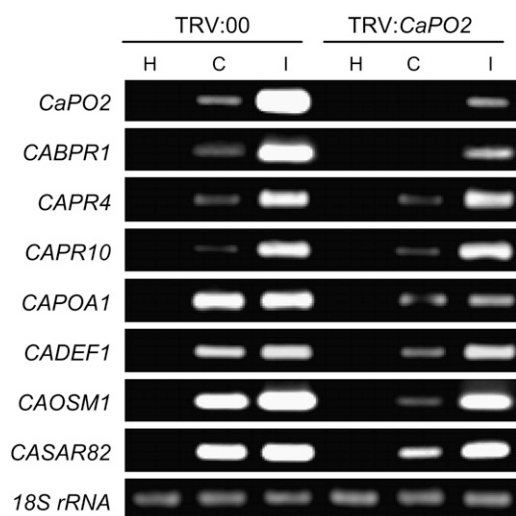


Figure 4. RT-PCR analysis of the expression of *CaPO2* and pepper defense-related genes in empty vector control (TRV:00) and *CaPO2* gene-silenced (TRV:*CaPO2*) pepper plants 24 h after inoculation with the virulent strain Ds1 (C, compatible) and the avirulent strain Bv5-4a (I, incompatible) of *Xcv* (5×10^6 cfu mL⁻¹). The level of 18S rRNA was visualized as a control. This experiment was repeated three times with similar results. H, Healthy leaves.

plants, we further analyzed the transcript levels of several defense-related pepper genes by RT-PCR (Fig. 4). The *CaBPR1*, *CaPR4* (putative antifungal protein), and *CaPR10* genes were strongly induced by avirulent *Xcv* infection in the empty vector control plants (TRV:00); however, induction of *CaBPR1* was compromised in *CaPO2*-silenced plants infected with either virulent or avirulent *Xcv*. Silencing of the *CaPO2* gene also remarkably compromised the induction of the *CaPOA1* (ascorbate peroxidase), *CaDEF1* (defensin), *CaOSM1* (osmotin-like protein), and *CaSAR82* (SAR8.2) genes by *Xcv* infection, especially by the virulent strain.

Silencing of the *CaPO2* gene led to a highly susceptible response to infection by the *Xcv* virulent strain Ds1 (5×10^6 colony forming units [cfu] mL⁻¹), accompanied by accelerated chlorosis (Fig. 5, A and B). *CaPO2*-silenced pepper leaves (TRV:*CaPO2*) started to exhibit chlorosis in perivascular regions 3 d after *Xcv* inoculation, whereas the empty vector control leaves (TRV:00) became chlorotic 5 d after inoculation. *CaPO2*-silenced leaves exhibited more severe chlorosis phenotypes than did the empty vector control leaves 7 d after inoculation. In particular, inoculation with the virulent strain Ds1 resulted in high levels of bacterial growth in the silenced plants compared with control plants (Fig. 5C). These data suggest that the extracellular peroxidase gene *CaPO2* plays important roles in the basal resistance of pepper plants to virulent *Xcv* infection.

Silencing of the *CaPO2* Gene in Pepper Compromises Oxidative Bursts and HRs, Locally and Systemically

To investigate the roles of the *CaPO2* gene in the oxidative burst and HR induction, H₂O₂ production

and micro-HR formation in infected primary and uninfected secondary pepper leaves after inoculation with an avirulent *Xcv* strain were observed by staining with DAB and trypan blue. Silencing of the *CaPO2* gene compromised not only oxidative bursts and the HR in local infected leaves but also reduced micro-bursts and micro-HR in uninoculated secondary leaves 24 h after infection with the *Xcv* avirulent strain Bv5-4a (Fig. 6A). This result suggests that extracellular

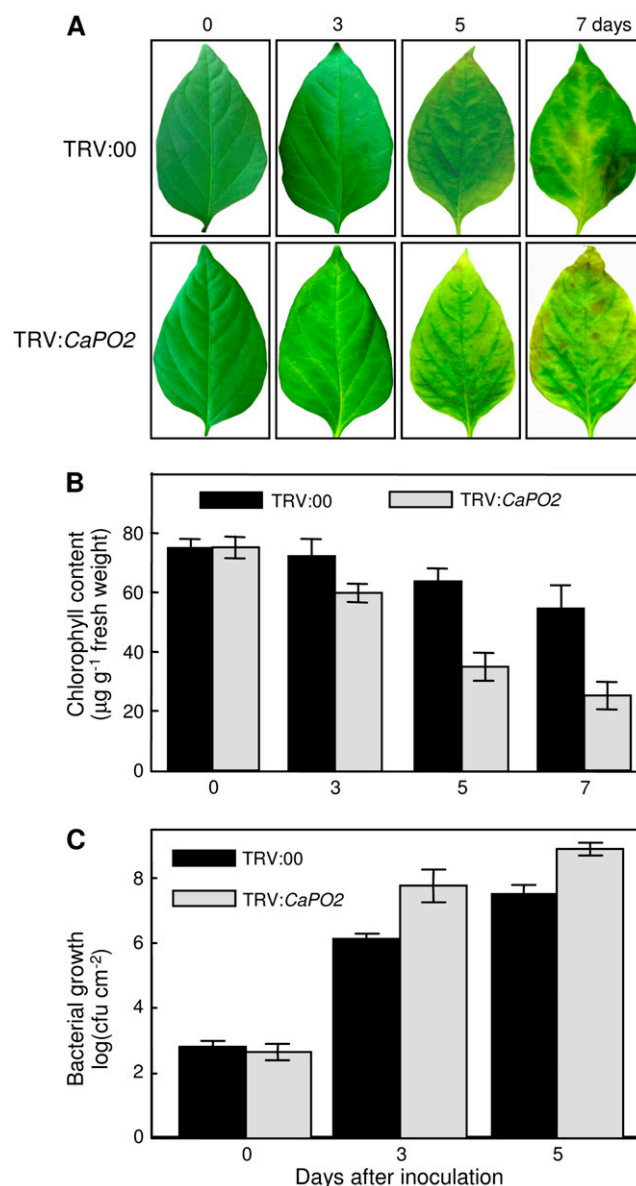


Figure 5. Enhanced disease susceptibility of *CaPO2* gene-silenced pepper plants to infection by the *Xcv* virulent strain Ds1. A, Representative disease symptoms developed on the leaves 0, 3, 5, and 7 d after inoculation (5×10^6 cfu mL⁻¹). B, Chlorophyll contents of empty vector control (TRV:00) or *CaPO2* gene-silenced (TRV:*CaPO2*) pepper plants 0, 3, 5, and 7 d after inoculation. C, Bacterial growth in leaves of the empty vector control (TRV:00) or *CaPO2* gene-silenced (TRV:*CaPO2*) pepper plants 0, 3, and 5 d after inoculation (10^4 cfu mL⁻¹). Data are the means \pm sds from three independent experiments.

peroxidase CaPO2-dependent ROS generation is required to generate oxidative bursts and induce the HR, both locally and systemically. Cell death was measured by ion leakage from leaf discs of empty vector control (TRV:00) and *CaPO2*-silenced (TRV:*CaPO2*) leaves inoculated with the avirulent strain Bv5-4a (Fig. 6B). The original conductivity strength in *CaPO2*-silenced pepper leaves was reduced by half compared with empty vector controls. In *CaPO2*-silenced leaves, small but significantly higher bacterial growth was observed 3 and 5 d after inoculation with the avirulent strain Bv5-4a as compared with TRV-infected control leaves (Fig. 6C), indicating that silencing of the *CaPO2* gene enhanced susceptibility to infection by the avirulent strain Bv5-4a.

Overexpression of *CaPO2* in Arabidopsis Enhances Disease Resistance Accompanied by Micro-HRs

Because transformation is very difficult in pepper plants, we overexpressed *CaPO2* in Arabidopsis to

determine the in planta functions of the gene. To generate transgenic Arabidopsis plants expressing the *CaPO2* gene, the *CaPO2* open reading frame was integrated between the CaMV 35S promoter and the *nos* terminator region in the binary vector pBIN35S (Supplemental Fig. S2A). Arabidopsis ecotype Columbia (Col-0) plants were transformed using the 35S:*CaPO2* construct according to the floral dipping method (Clough and Bent, 1998). Among 13 lines of *CaPO2*-OX plants, the *CaPO2*-OX mutant T₂ lines 2, 11, and 13, which strongly expressed the *CaPO2* gene, were selected for further experiments (Supplemental Fig. S2B). We did not observe any apparent phenotypic differences between wild-type and *CaPO2*-OX mutant lines. Wild-type and *CaPO2*-OX plants were evaluated for total peroxidase activity as described by Hammerschmidt et al. (1982; Supplemental Fig. S2C). Wild-type plants contained a low level of peroxidase activity, but all tested *CaPO2*-OX plants (T₂) exhibited a 3- to 5-fold higher total peroxidase activity than wild-type plants. This result suggests that overexpression of

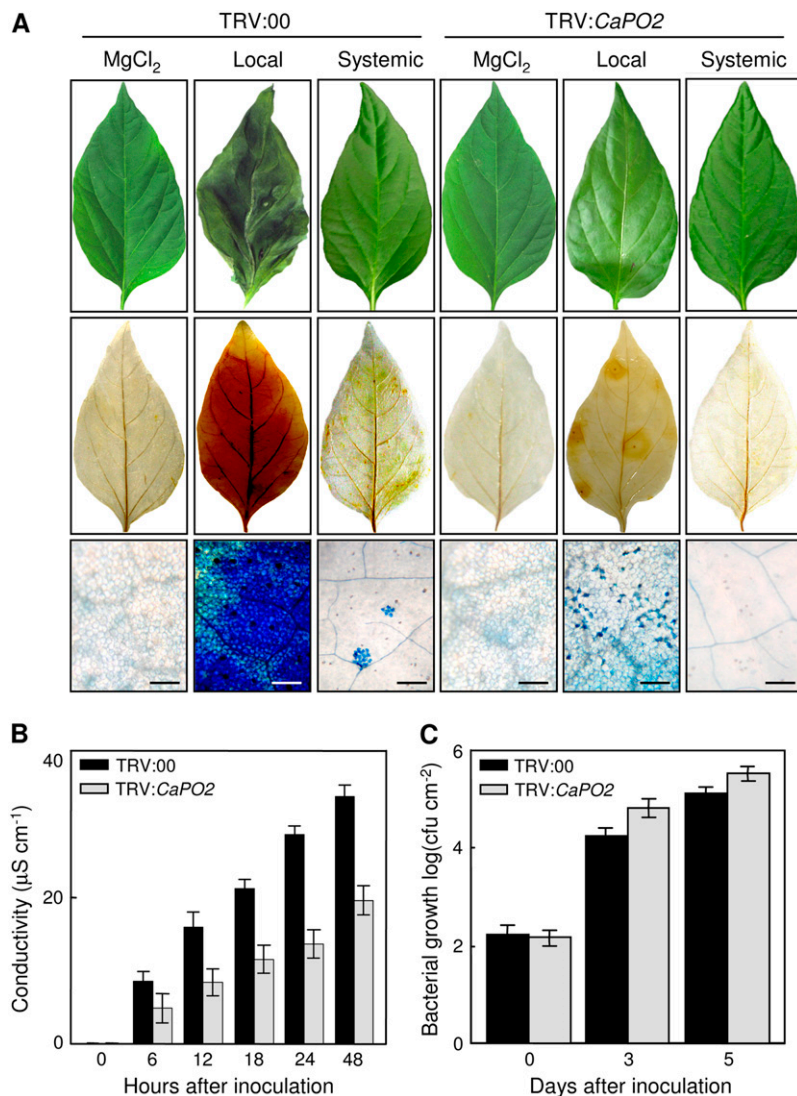


Figure 6. Reduction of the oxidative burst and HR phenotypes in the *CaPO2* gene-silenced pepper leaves infected by the *Xcv* avirulent strain Bv5-4a. **A**, Representative HR symptoms developed on the leaves 24 h after inoculation (5×10^6 cfu mL⁻¹). *Xcv*-infected primary leaves were harvested and stained with DAB and trypan blue 1 and 24 h after inoculation, respectively; however, uninoculated secondary leaves were stained with both agents 24 h after inoculation. Bars = 200 μm. **B**, Cell death measured as ion leakage from leaf discs of empty vector control (TRV:00) and *CaPO2* gene-silenced (TRV:*CaPO2*) pepper plants at various times after inoculation (5×10^6 cfu mL⁻¹). Data are the means \pm sds from three independent experiments. **C**, Bacterial growth in empty vector control (TRV:00) or *CaPO2* gene-silenced (TRV:*CaPO2*) pepper leaves 0, 3, and 5 d after inoculation (10^4 cfu mL⁻¹). Data are the means \pm sds from three independent experiments.

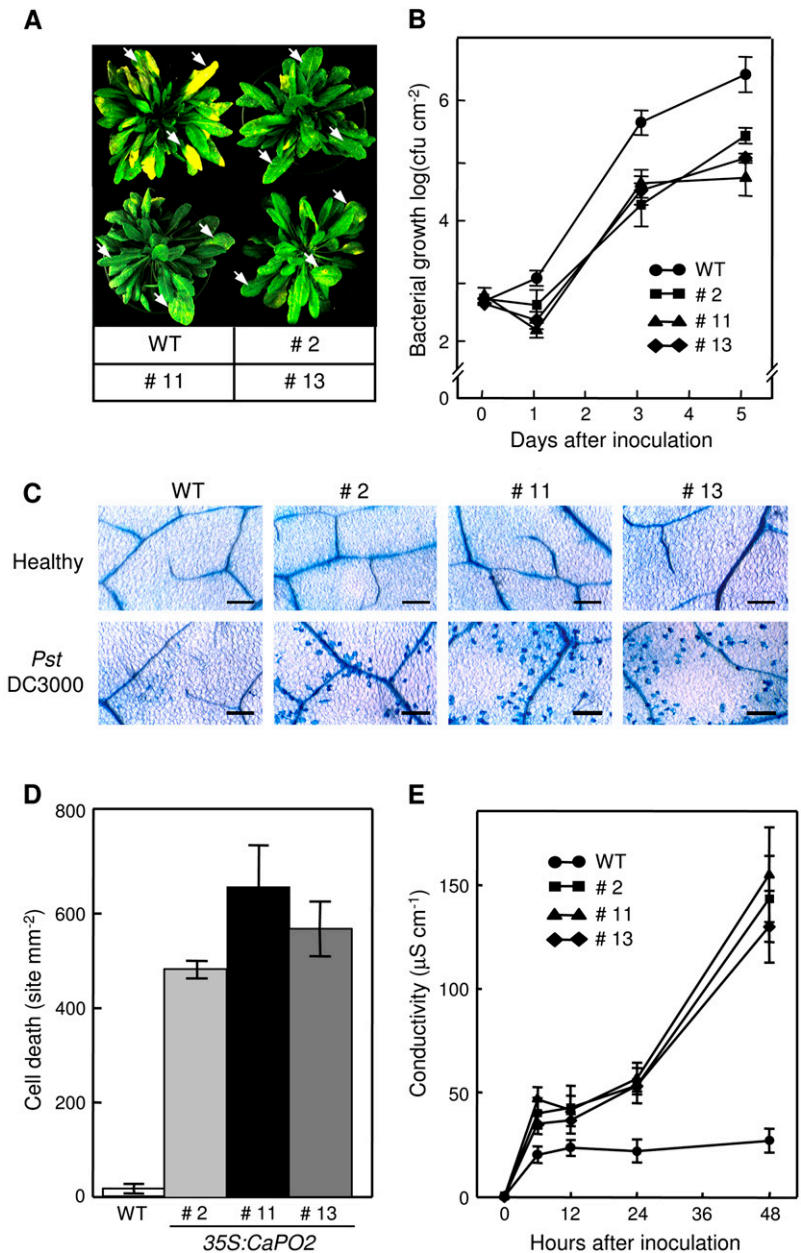
the *CaPO2* gene in Arabidopsis may enhance peroxidase activity.

In the loss-of-function analysis, silencing of the *CaPO2* gene increased susceptibility to *Xcv* infection, which suggested a possible role for *CaPO2* in the defense response of pepper plants to bacterial pathogen infection (Figs. 5 and 6). For the gain-of-function analysis, we evaluated whether overexpression of the *CaPO2* gene in Arabidopsis confers enhanced resistance to *P. syringae* pv *tomato* (*Pst*) DC3000 infection. Seven days after inoculation, disease symptoms developed on the leaves of wild-type plants but not on *CaPO2*-OX plants (Fig. 7A). All *CaPO2*-OX lines exhibited slightly decreased bacterial growth 1 d after inoculation, compared with wild-type plants. Three

and 5 d after inoculation, however, all *CaPO2*-OX mutant plants examined exhibited an approximately 10-fold reduction in bacterial growth as compared to wild-type plants (Fig. 7B). Together, these findings suggest that overexpression of the *CaPO2* gene enhances the basal resistance of Arabidopsis plants to *Pst* DC3000 infection.

Arabidopsis *CaPO2*-OX leaves infiltrated with 10^6 cfu mL⁻¹ of *Pst* DC3000 (empty vector) exhibited numerous micro-HRs that left no detectable symptoms (Fig. 7, C and D). In contrast, a faint and negligible cell death response was observed in the leaves of wild-type plants following *Pst* DC3000 infection. Microscopic observations of trypan blue-stained leaves revealed that many cell death sites were located in

Figure 7. Enhanced resistance of Arabidopsis *CaPO2*-OX plants to *Pst* DC3000 infection accompanied by micro-HRs. A, Disease symptoms developed on the leaves of wild-type and *CaPO2*-OX plants 7 d after inoculation (10^4 cfu mL⁻¹). Arrows indicate infected leaves. B, Bacterial growth in the leaves of wild-type and *CaPO2*-OX plants 0, 1, 3, and 5 d after inoculation (10^4 cfu mL⁻¹). Data are the means \pm sds from three independent experiments. C, Trypan blue staining of micro-HRs in leaf tissues of *CaPO2*-OX plants 24 h after inoculation (10^6 cfu mL⁻¹). Bars = 200 μ m. D, Quantification of cell death. Clusters of dead cells were identified and quantified by the trypan blue staining of wild-type and *CaPO2*-OX leaves 24 h after inoculation (10^6 cfu mL⁻¹). Data are the means \pm sds from three independent experiments. E, Ion leakage from leaf discs of wild-type and *CaPO2*-OX plants 0, 12, 24, 36, and 48 h after inoculation (10^6 cfu mL⁻¹). Data are the means \pm sds from three independent experiments.



periveinal regions in the leaves of *CaPO2*-OX plants (Fig. 7C). Consistent with the result of the trypan blue staining, *CaPO2*-OX plants exhibited significantly greater ion leakage levels compared with wild-type plants (Fig. 7E). Together, these results suggest that ectopic expression of *CaPO2* in *Arabidopsis* increases basal resistance accompanied by a micro-HR response to virulent *Pst* DC3000 infection.

Overexpression of *CaPO2* in *Arabidopsis* Increases H_2O_2 Generation and *PR* Gene Induction in Response to Virulent *Pst* DC3000 Infection

To determine whether the *CaPO2* peroxidase acts as a ROS-detoxifying or -generating enzyme during pathogenesis, leaves of *Arabidopsis* wild-type and *CaPO2*-OX plants were stained with DAB after infiltration with *Pst* DC3000 (10^6 cfu mL⁻¹). Wild-type leaves inoculated with *Pst* DC3000 were only faintly colored reddish brown by the DAB polymer. The leaves of *Arabidopsis CaPO2*-OX lines that constitutively expressed the transgene were stained a vivid reddish brown after inoculation with *Pst* DC3000 as compared with wild-type plants (Fig. 8). This result suggests that ectopic expression of *CaPO2* in *Arabidopsis* induces H_2O_2 generation in response to *Pst* DC3000 infection. To determine whether the H_2O_2 generation is induced by the *Pst* DC3000 infection in a peroxidase-independent manner, we further quantified the H_2O_2 amounts using the xylenol orange assay (Gay et al., 1999; Bindschedler et al., 2001; Bindschedler et al., 2006; Supplemental Fig. S3). Consistent with the DAB staining results, *CaPO2*-OX induced higher levels of H_2O_2 in leaf tissues of all tested transgenic lines early after inoculation with *Pst* DC3000 compared to wild-type plants (Supplemental Fig. S3A).

To confirm whether the generation of H_2O_2 is induced by the *CaPO2* protein, leaves of wild-type and *CaPO2*-OX plants were infiltrated with 10^6 cfu mL⁻¹ of *Pst* DC3000 and supplemented with 1 mM KCN or 2.5 μ M DPI (Fig. 8). In general, KCN and DPI inhibit peroxidase and NADPH oxidase activities, respectively (Pellinen et al., 1999; Orozco-Cárdenas et al., 2001; Zhang et al., 2001; Romero-Puertas et al., 2004). Coinfiltration of *Pst* DC3000 with 1 mM KCN effectively inhibited the *CaPO2*-mediated generation of H_2O_2 in leaves of *CaPO2*-OX plants, whereas treatment with 2.5 μ M DPI did not. No changes in H_2O_2 accumulation were observed in wild-type plants infiltrated with 1 mM KCN or 2.5 μ M DPI. Furthermore, similar results were observed by xylenol orange assay (Supplemental Fig. S3B). Coinfiltration of *Pst* DC3000 with 1 mM KCN, but not with 2.5 μ M DPI, significantly lowered the H_2O_2 induction in leaves of *CaPO2*-OX line (no. 11). These findings suggest that H_2O_2 accumulation in *CaPO2*-OX plants after *Pst* DC3000 infection is *CaPO2* peroxidase dependent but not NADPH oxidase independent.

PR gene expression in *Arabidopsis* wild-type and *CaPO2*-OX plants was analyzed by RT-PCR and real-time RT-PCR (Fig. 9, A and B). The *Arabidopsis PR*

genes examined were not expressed in healthy leaves of wild-type or *CaPO2*-OX plants. However, *Pst* DC3000 infection differentially induced the *Arabidopsis PR* genes *NPR1*, *PR1*, and *PR5*, which are known to be regulated by the salicylic acid (SA)-dependent pathway in *CaPO2*-OX plants compared with wild-type plants. In wild-type plants, *PR1* and *PR5* were slightly induced by mock and *Pst* DC3000 infection. However, *CaPO2*-OX plants exhibited a rapid and significantly enhanced induction of *NPR1*, *PR1*, and *PR5* after mock and *Pst* DC3000 infection. The *CaPO2*-OX mutant line 11, which showed the highest expression level of *CaPO2* and peroxidase activities, exhibited higher levels of induced *NPR1*, *PR1*, and *PR5* than did the *CaPO2*-OX lines 2 and 13 (Fig. 9, A and B). This result suggests that ectopic expression of the *CaPO2* gene in *Arabidopsis* triggers the SA-dependent defense pathway during virulent *Pst* DC3000 infection.

Overexpression of *CaPO2* in *Arabidopsis* Accelerates Cell Death against Avirulent *Pst* DC3000 (*avrRpm1* or *avrRpt2*) Infection

To determine whether *CaPO2* overexpression in *Arabidopsis* influences the HR, wild-type and *CaPO2*-OX plants were infiltrated with avirulent *Pst* DC3000 carrying *avrRpm1* or *avrRpt2*, followed by trypan blue staining and ion conductivity measurement. *CaPO2*-OX lines infiltrated with avirulent *Pst* DC3000 (*avrRpm1* or *avrRpt2*) exhibited significantly accelerated and increased cell death phenotypes compared with wild-type plants (Fig. 10A). Consistent with the results of trypan

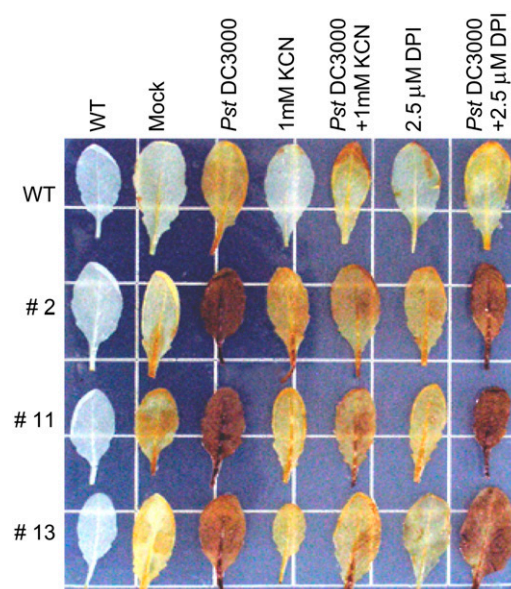


Figure 8. Detection of H_2O_2 in *Arabidopsis* wild-type and *CaPO2*-OX leaves by staining with DAB 10 min after infiltration with *Pst* DC3000 (empty vector; 10^6 cfu mL⁻¹) in the presence or absence of 1 mM KCN (peroxidase inhibitor) or 2.5 μ M DPI (NADPH oxidase complex inhibitor). WT, Wild type; Mock, mock treated.

blue staining, inoculation with avirulent *Pst* DC3000 (*avrRpm1* or *avrRpt2*) resulted in a significant increase in ion conductivity levels in *CaPO2*-OX plants, as compared to wild-type plants (Fig. 10B).

PR1 induction by *Pst* DC3000 (*avrRpm1* or *avrRpt2*) infection in wild-type and *CaPO2*-OX plants was monitored by RNA gel-blot analysis (Fig. 10C). There was no significant difference in *PR1* expression between wild-type and *CaPO2*-OX plants 12 h after inoculation with *Pst* DC3000 carrying *avrRpm1*. However, *PR1* was rapidly induced in *CaPO2*-OX plants challenged with avirulent *Pst* DC3000 (*avrRpt2*) compared to wild-type plants.

Overexpression of *CaPO2* in Arabidopsis Enhances Systemic Microbursts and Micro-HRs against *Pst* DC3000 Infection

To determine whether overexpression of the *CaPO2* gene increases microbursts and micro-HRs in uninoculated secondary leaves of Arabidopsis plants, primary and secondary leaves were stained with DAB and trypan blue 24 h after inoculation of primary leaves with *Pst* DC3000 (empty vector or *avrRpt2*; Fig. 11). Infection by avirulent *Pst* DC3000 (*avrRpt2*) resulted in strong oxidative bursts and hypersensitive cell death in inoculated primary leaves, as well as significant microbursts and micro-HRs in uninoculated secondary leaves. However, we did not observe microbursts or the HRs in the leaves of wild-type plants infected with virulent *Pst* DC3000. The induction of microbursts and micro-HRs was significantly greater in the secondary leaves of Arabidopsis *CaPO2*-OX plants compared with wild-type plants. The systemic microbursts and micro-HRs in wild-type and *CaPO2*-OX leaves were eliminated by infiltration of secondary leaves with KCN and DPI. These findings suggest that the ectopic expression of *CaPO2* enhances the level of systemic microbursts and micro-HR foci in response to *Pst* DC3000 infection.

DISCUSSION

In a search for defense-related genes in a pepper cDNA library, we observed that the pepper extracellular peroxidase *CaPO2* gene is locally or systemically induced by avirulent *X. campestris* pv. *vesicatoria* infection. In addition, staining with DAB and trypan blue revealed that avirulent *Xcv* infection strongly induces oxidative bursts and hypersensitive cell death, as well as *CaPO2* expression, both locally and systemically. These findings allowed us to test whether the *CaPO2* gene mediates oxidative bursts and cell death in both primary and secondary leaves of pepper plants in response to *Xcv* infection.

We used VIGS to dissect the biological functions of the *CaPO2* gene in generating oxidative bursts and promoting cell death during the defense response in pepper plants. *CaPO2*-silenced plants were susceptible to viru-

lent *X. campestris* pv. *vesicatoria*, which resulted in enhanced bacterial growth and accelerated chlorosis. Furthermore, VIGS of the *CaPO2* gene also compromised oxidative burst generation and hypersensitive cell death during avirulent *Xcv* infection. These findings suggest that *CaPO2*-mediated ROS generation plays a pivotal role in basal resistance, as well as in the HR.

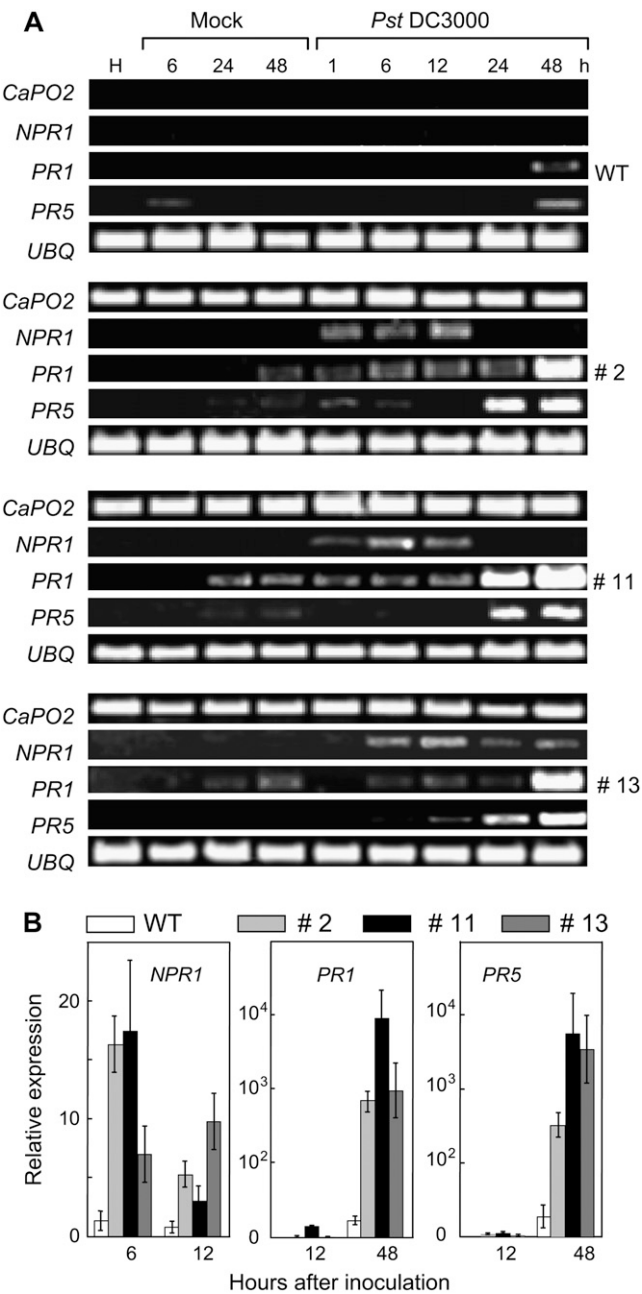


Figure 9. Accelerated and increased expression of Arabidopsis *PR* genes in *CaPO2*-OX lines after infection with *Pst* DC3000. RT-PCR (A) and real-time RT-PCR (B) analyses of expression of the *NPR1*, *PR1*, and *PR5* genes in Arabidopsis wild-type and *CaPO2*-OX plants at different times after inoculation (10^5 cfu mL⁻¹). Expression of the *UBQ* gene served as a control. The data in B are the means \pm SD from three independent experiments.

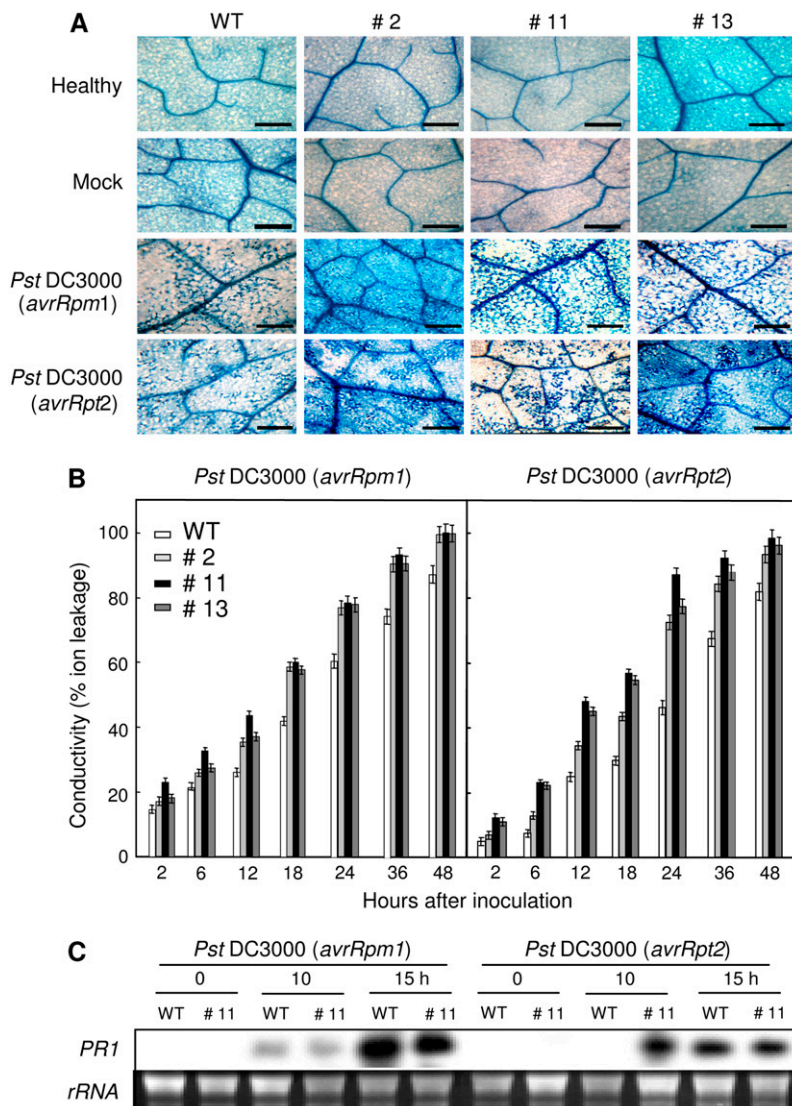


Figure 10. Accelerated HRs of Arabidopsis *CaPO2*-OX plants to *Pst* DC3000 carrying *avrRpm1* or *avrRpt2*. A, Trypan blue staining to detect the HR in leaf tissues of wild-type and *CaPO2*-OX plants 24 h after inoculation (10^6 cfu mL $^{-1}$). B, Cell death measured as ion leakage from leaf discs of wild-type and *CaPO2*-OX plants at various times after inoculation (10^6 cfu mL $^{-1}$). Data are the means \pm sds from three independent experiments. C, RNA gel-blot analysis of *PR1* expression in wild-type and *CaPO2*-OX plants at various times after inoculation (10^6 cfu mL $^{-1}$). The membrane was hybridized with a 32 P-dCTP-labeled *PR1* probe. Equal loading of total RNA (10 μ g per lane) was verified by visualizing rRNA on a gel stained with ethidium bromide. WT, Wild type; #2, #11, and #13, Arabidopsis *CaPO2*-OX lines. Experiments were repeated three times with similar results.

Multigene families of peroxidases are involved in various physiological processes in plants, and members of these families exhibit diverse expression patterns under stressful conditions (Hiraga et al., 2001; Passardi et al., 2004, 2005). These variable expression patterns are suggestive of pivotal roles for peroxidases in plant life cycles. Several peroxidase genes are expressed not only in response to pathogen attack but are also involved in the cross-linking of cell wall proteins or the generation of ROS. Growing numbers of publications have reported significant roles for peroxidases in defense responses (Hilaire et al., 2001; De Biasi et al., 2003; Bindschedler et al., 2006). Infection of rice leaves by *Xanthomonas oryzae* pv *oryzae*, the causal pathogen of rice blight, strongly induces a peroxidase isoform in xylem vessels, which results in secondary wall thickening and reduced access of the pathogen to the pit membrane, which is the pathogen's contact point in living cells (Hilaire et al., 2001). In tomato (*Solanum lycopersicum*) plants, H₂O₂ generation by cell

wall peroxidase was demonstrated to be involved in the cell death response controlled by the Pto-Fen complex rather than by NADPH oxidases (De Biasi et al., 2003). More recently, studies with antisense *FRENCH BEAN PEROXIDASE1* in transgenic Arabidopsis plants have suggested significant roles for peroxidases in the apoplastic oxidative burst and plant defense response (Bindschedler et al., 2006). However, the function of such extracellular peroxidases in hypersensitive cell death is not fully understood. In this study, we revealed biological functions for a novel pepper extracellular peroxidase gene—the *CaPO2*-dependent generation of ROS in hypersensitive cell death—by analysis of *CaPO2*-silenced pepper plants and *CaPO2*-OX transgenic Arabidopsis. Silencing of the *CaPO2* gene not only enhanced susceptibility to *Xcv* infection but also compromised H₂O₂ accumulation and hypersensitive cell death in pepper leaves without inactivation of the NADPH oxidase complex in response to the *Xcv* avirulent strain Bv5-4a. Furthermore,

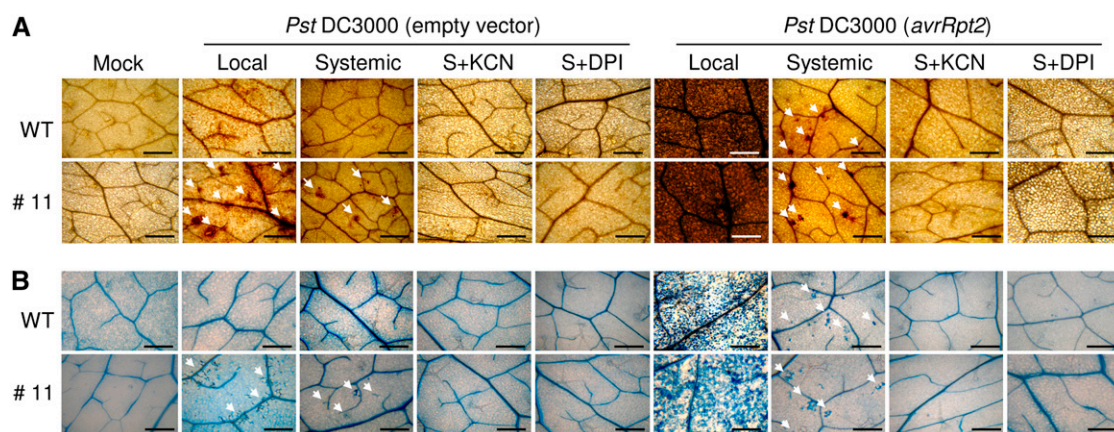


Figure 11. Enhanced SAR phenotypes in Arabidopsis *CaPO2*-OX plants. DAB (A) and trypan blue (B) staining of primary and secondary leaves of wild-type and *CaPO2*-OX plants 24 h after challenging lower primary leaves with *Pst* DC3000 or *Pst* DC3000 (*avrRpt2*; 10^7 cfu mL⁻¹). Secondary (S) leaves were also simultaneously infiltrated with 1 mM KCN (peroxidase inhibitor) or 2.5 μM DPI (NADPH oxidase complex inhibitor) immediately after *Pst* inoculation. Infected local leaves were stained with DAB and trypan blue 10 min and 24 h after inoculation, respectively. Arrows indicate microbursts and micro-HRs. Bars = 500 μm.

strong H₂O₂ and micro-HR formation was detected in *CaPO2*-OX Arabidopsis plants after infection by a virulent strain of *Pst* DC3000, compared to wild-type Arabidopsis plants. These findings strongly support the notion that the *CaPO2*-dependent generation of ROS has a pivotal role in hypersensitive cell death.

The oxidative burst is essential for the establishment of plant immunity (Alvarez et al., 1998; Kim and Martin, 2004; Park, 2005). To determine whether *CaPO2*-mediated ROS accumulation and micro-HR formation are associated with the activation of *PR* gene expression, we carried out RT-PCR and real-time RT-PCR analyses in Arabidopsis. Strikingly, these analyses revealed that *CaPO2*-OX lines exhibited remarkably enhanced levels of *PR1* and *PR5* gene expression after inoculation with virulent *Pst* DC3000 (no *avr* gene). *PR1* and *PR5* genes are known as SA pathway-dependent defense-related genes. In particular, *NPR1* (Arabidopsis nonexpressor of pathogenesis-related gene), a positive regulator of *PR* gene expression, was also detected in *CaPO2*-OX lines infected by virulent *Pst* DC3000. Furthermore, silencing of the *CaPO2* gene significantly lowered *CaBPR1* expression levels in pepper plants after *Xcv* infection. This finding suggests that *CaPO2*-mediated ROS accumulation stimulates *PR* gene expression. Silencing of *CaPO2* also lowered the level of *CaPOA1* gene expression after *Xcv* infection. Ascorbate peroxidase is known as a major ROS scavenging enzyme during the oxidative stresses (Muraglia et al., 2004). The lowered levels of *CaPOA1* transcript are consistent with the lowered oxidative burst levels in *CaPO2*-silenced pepper plants during pathogen infection. Therefore, we conclude that the *CaPO2*-mediated oxidative burst during pathogen infection triggers *PR* gene induction.

CaPO2-OX plants exhibited a significantly accelerated cell death response to infection by *Pst* DC3000 carrying *avrRpm1* or *avrRpt2*. Moreover, *PR1* induction

was accelerated in Arabidopsis *CaPO2*-OX plants infected by *Pst* DC3000 carrying *avrRpt2*. The *P. syringae* type III effectors *AvrRpm1* and *AvrRpt2* have been demonstrated to posttranscriptionally phosphorylate and eliminate the RIN4 protein, thereby triggering RPM1 (resistance to *P. syringae* pv *maculicola* 1)- and RPS2-mediated defense signaling, respectively (Mackey et al., 2003). However, the downstream signaling and defense mechanism of RPM1 and RPS2 is not fully understood. Therefore, the relationship of the *avrRpt2*-specific accelerated cell death to *PR1* gene expression in *CaPO2*-OX plants should be elucidated.

The generation of ROS (O₂⁻ and H₂O₂) is closely related to plant defense responses, especially the HR (Apel and Hirt, 2004; Wagner et al., 2004; Yun et al., 2006) and SAR (Alvarez et al., 1998). In earlier studies, the Arabidopsis NADPH oxidase was demonstrated to induce ROS during systemic resistance in the presence of an NADPH oxidase complex inhibitor (DPI) and catalase (Alvarez et al., 1998). However, the extracellular peroxidase-dependent generation of ROS during SAR has not yet been reported. To investigate whether pepper plants harbor NADPH oxidase complex- or peroxidase-dependent ROS generation systems in systemic microbursts and micro-HR foci, we used KCN and DPI as peroxidase and NADPH oxidase complex inhibitors, respectively. Treatment of secondary pepper leaves with both KCN and DPI eliminated microburst and micro-HR phenotypes induced by the *Xcv* avirulent strain Bv5-4a, accompanied by the elimination of *CaPO2* gene expression. Moreover, silencing of the *CaPO2* gene also compromised systemic microbursts and micro-HRs in pepper plants inoculated with the *Xcv* avirulent strain Bv5-4a. Interestingly, significant microbursts and micro-HRs were observed in the secondary leaves of *CaPO2*-OX plants but not of wild-type plants during virulent *Pst* DC3000 infection. These findings support the notion

that the CaPO2 and NADPH oxidases are required for microbursts and micro-HRs in uninoculated secondary leaves of pepper plants. To the best of our knowledge, this is the first report noting the induction of the systemic microburst and micro-HR phenotype by extracellular peroxidase-dependent ROS generation. The induction of the oxidative burst and of defense-related genes such as *CaPO2* in uninoculated secondary leaves may be essential for establishing SAR in pepper plants, as previously suggested (Lee and Hwang, 2005).

In human leukocytes activating immune response to pathogen infection, the heme-containing myeloperoxidase (MPO) is secreted at sites of infection and is involved in ROS generation and tissue damage (Arnhold, 2004). In particular, hereditary MPO deficiency leads to a recessive leukocyte disorder associated with defective candidacidal activity and increased susceptibility to pathogen infection. This observation suggests that MPO plays a significant role in the human defense system. Our primary concern was to determine how heme-containing pepper extracellular peroxidases CaPO2 contribute to defense responses of pepper plants to pathogen infection. Our results suggest pivotal roles for the extracellular peroxidase CaPO2 in oxidative burst generation, cell death, and *PR* gene induction for disease resistance in plants via the following mechanisms. First, recognition of pathogen invasion (especially by avirulent strains) induces *CaPO2* gene expression in pepper plants. Second, CaPO2 protein is required for early H₂O₂ generation during pathogen infection. Arabidopsis *CaPO2* transgenic plants that constitutively overexpress *CaPO2* did not accumulate ROS in the absence of pathogen infection. However, significant ROS accumulation and micro-HR formation were detected during *Pst* DC3000 infection, especially avirulent *Pst* infection. Finally, CaPO2-dependent ROS generation activates defense responses, including hypersensitive cell death, *PR* gene induction, and SAR. The extracellular peroxidase CaPO2 and NADPH oxidase-mediated ROS generation is required for systemic microbursts and micro-HRs, which may contribute to the establishment of SAR. In principle, plants harbor two innate immune systems (Jones and Dangl, 2006). The first is a basal resistance which uses transmembrane pattern recognition receptors for the detection of slowly evolving pathogen-associated molecular patterns of pathogens. The second is a HR that uses R-protein-mediated recognition of avirulence proteins from avirulent pathogens. Taken together, our data suggest that CaPO2-mediated ROS generation is required for both basal resistance and HR in plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Pepper (*Capsicum annuum* 'Nockwang') plants were grown in a plastic tray (55 × 35 × 15 cm) containing steam-sterilized soil mix (peat moss, perlite, and vermiculite; 5:3:2, v/v/v) and loam soil (1:1, v/v) at 28°C with a day length of

16 h at a light intensity of 70 μmol photons m⁻²s⁻¹. Six seedlings at the two-leaf stage were transplanted to a plastic pot (5 × 15 × 10 cm) containing the same soil mix. Pepper plants at the six-leaf stage were used for pathogen infection and abiotic elicitor and environmental stress treatment.

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 plants were grown at 24°C with a photosynthetic flux of 130 μmol photons m⁻² s⁻¹ for 16 h (long day condition) or 12 h (short day condition) light and 60% relative humidity in a controlled environmental chamber. Plants were raised in pots containing vermiculite, peat moss, and perlite (1:1:0.5, v/v/v). Prior to sowing, the seeds of wild-type (Col-0) and *CaPO2*-OX plants were sterilized with a 2% sodium hypochlorite solution, followed by imbibition at 4°C for 3 d to overcome dormancy.

Pathogens and Inoculation Procedures

The virulent strain Ds1 and the avirulent strain Bv5-4a of *Xcv* were used in this study. Bacteria were cultured overnight in yeast-nutrient broth (5 g yeast [*Saccharomyces cerevisiae*] extract, 8 g nutrient broth L⁻¹) at 28°C. Prior to inoculation, bacterial cells were collected by centrifugation and resuspended in 10 mM MgCl₂ solution (10⁸ cfu mL⁻¹). Pepper plants at the six-leaf stage were inoculated by infiltrating the bacterial suspension into the abaxial side of fully expanded leaves using a syringe without a needle. The infected plants were incubated in a controlled chamber at 28°C with 100% relative humidity for 16 h. Infected primary and uninfected secondary upper leaves were harvested at various time points for bacterial growth assays, RNA isolation, and histochemical analysis.

Pst DC3000 was grown overnight in King's B medium containing 50 μg mL⁻¹ rifampicin. To determine bacterial growth, leaves of 4-week-old wild-type and *CaPO2* transgenic Arabidopsis T₂ plants were infiltrated with 10⁴ cfu mL⁻¹ of *Pst* DC3000 in 10 mM MgCl₂ using a syringe without a needle. Infected leaves were harvested 0, 1, 3, and 5 d after inoculation. Bacterial growth experiments were repeated three times with similar results.

Isolation and Sequence Analysis of Pathogen-Induced *CaPO2* cDNAs

For construction of pathogen-induced cDNA library, the avirulent strain Bv5-4a of *Xcv* was used to inoculate pepper leaves. The pepper cDNA library was constructed using 5 μg poly(A)⁺ mRNA extracted from inoculated pepper leaves (Kim and Hwang, 2000). To isolate pathogen-inducible cDNAs from the pepper cDNA library, differential hybridization was performed according to the method of Jung and Hwang (2000). Among the cDNA clones tested, the *CaPO2* cDNA clone hybridized strongly and differentially to the cDNA probes from leaves infected by the avirulent strain Bv5-4a of *Xcv*.

Particle Bombardment

The coding region of the *CaPO2* gene was cloned between the *CaMV* 35 promoter and *smGFP* region of the binary vector p326GFP to generate a C-terminal fusion of *smGFP* to *CaPO2*. For particle bombardment, the plasmids were purified using QIAGEN plasmid maxi kits according to the manufacturer's instructions (Qiagen). Onion (*Allium cepa*) epidermis was bombarded with gold particles coated with plasmids using a Bio-Rad (Hercules) PDS-1000/He particle delivery system. Bombarded specimens were incubated for 24 h on 1 × Murashige and Skoog agar media and observed using a MRC-1024 confocal laser-scanning microscope (Bio-Rad).

RNA Isolation and RNA Gel-Blot Analysis

Total RNA was extracted from pepper leaves, stems, roots, flowers, and fruits using the guanidine isothiocyanate method (Chomczynski and Sacchi, 1987). Total cellular RNA of *CaPO2*-OX Arabidopsis T₂ plants was also extracted from the aerial portions of plants using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. To generate a gene-specific probe, the coding region of the *CaPO2* gene was amplified using the primers *CaPO2*F (5'-ATGGCAGAGAAAACCACAGCA-3') and *CaPO2*R (5'-TCA-AAAAAAGTGACCTCTTCTGT-3'). The amplified PCR product was ³²P labeled using a random priming kit (Boehringer Mannheim). Agarose gel electrophoresis, RNA transfers, and hybridization with the *CaPO2* fragment were performed using standard procedures.

VIGS

The TRV-based VIGS system was used for gene silencing in pepper plants as described by Liu et al. (2002). The coding region of the pepper *CaPO2* gene was cloned into the vector pTRV2 to yield pTRV2:CaPO2. *Agrobacterium tumefaciens* strain GV3101 carrying pTRV1 or pTRV2:CaPO2 was coinfiltrated into the fully expanded cotyledons of pepper plants ($OD_{600} = 0.2$). Plants were placed in a greenhouse at 25°C with a 16-h light and 8-h dark photoperiod cycle to allow growth and viral spread.

Arabidopsis Transformation

Transgenic Arabidopsis plants expressing the *CaPO2* gene were generated using the floral dipping method (Clough and Bent, 1998). Thirteen lines of putative transgenic Arabidopsis plants (T_1) harboring the 35S:CaPO2 construct were selected by planting seeds on Murashige and Skoog plates (Duchefa) containing 50 mg L⁻¹ kanamycin. Three T_2 lines, 2, 11, and 13, were used for further study.

Histochemistry

DAB staining was performed as described by Thordal-Christensen et al. (1997). Inoculated leaves were detached at various time intervals and placed in 1 mg mL⁻¹ DAB (Sigma) solution for 15 h. To develop the redish-brown coloration of the DAB polymer, stained leaves were placed in boiling 95% ethanol for 10 min.

Trypan blue staining was performed as described by Koch and Slusarenko (1990). Infected leaves were sampled at the indicated time points after inoculation and stained with lactophenol-trypan blue solution (10 mL lactic acid, 10 mL glycerol, 10 g phenol, and 10 mg trypan blue, dissolved in 10 mL distilled water). Whole leaves were boiled for approximately 1 min in the staining solution and then decolorized in chloral hydrate (2.5 g chloral hydrate dissolved in 1 mL distilled water). They were mounted in 60% glycerol and representative phenotypes were photographed with a light microscope (Olympus, BH-2).

Real-Time RT-PCR and RT-PCR Analysis

The expression of the Arabidopsis *NPR1*, *PR1*, and *PR5* genes during *Pst* DC3000 infection was examined using the real-time RT-PCR technique. Wild-type and *CaPO2*-OX Arabidopsis transgenic leaves were inoculated with 10⁵ cfu mL⁻¹ of *Pst* DC3000 and sampled 1, 6, 12, 24, and 48 h after inoculation. For mock treatment, 10 mM MgCl₂ was used. Total RNA was extracted from the aerial portion of Arabidopsis T_2 plants using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (2 µg), avian myeloblastosis virus reverse transcriptase (Roche), and oligo p(dT)₁₅ primer (Roche) were used for cDNA synthesis. Gene-specific primer pairs for each amplification were: 5'-ATAGAGGACACATTGGTTATACTCAAGC-3' and 5'-GATCGAGCAGCGTCATCTTCAATTCAT-3' for *NPR1*, 5'-GTAGGTGCTCTTGTTCTTCCC-3' and 5'-GTATGGCTTCTCGTTCACATAATTC-3' for *PR1*, 5'-TTCACATCTCTTCTCGTGTCA-3' and 5'-TCGTAGTTAGCTCCGGTACAAAGTG-3' for *PR5*, and 5'-CAAGACAGGAGAAATATGTCTCG-3' and 5'-ATCCTTTCTTAGGCATAGCG-3' for *UBQ*. For real-time RT-PCR analysis, the SYBR Green Supermix (Bio-Rad) was used according to the manufacturer's instructions.

Ion Leakage Measurement

Cell death in wild-type, *CaPO2*-silenced, and *CaPO2*-OX plants was assessed by ion leakage measurement after inoculation with *Xcv* (5×10^6 cfu mL⁻¹) or *Pst* DC3000 (10^6 cfu mL⁻¹). Six leaf discs of known area (0.6 cm in diameter) were removed and washed three times with distilled water following infiltration and incubated in 3 mL distilled water at room temperature. The conductivity of the incubation medium was recorded at various time points with a Crison conductivity meter (Net InterLab). The results from three independent experiments are represented as means \pm sds.

Assay of Peroxidase Activity

To assess the total peroxidase activity, total proteins in wild-type and the *CaPO2*-OX plants were extracted by homogenizing the leaves with mortar and

pestle added with 5 volumes of extraction buffer (0.1 M sodium phosphate buffer, pH 6.0, 0.5 M Suc). The homogenates were centrifuged for 20 min at 10,000g and 4°C. The clear supernatant was used for peroxidase activity assay according to the method of Hammerschmidt et al. (1982). Peroxidase activity was determined using guaiacol as the hydrogen donor. Leaf extract (100 µL) was added to the reaction mix (0.1 M sodium phosphate buffer, pH 6.0, 0.25% guaiacol, 1 M H₂O₂), followed by incubation for 5 min at 25°C. Peroxidase activity was determined by an increase in $A_{470} \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$. Peroxidase activity of leaf extracts was calculated using the molar extinction coefficient of tetraguaiacol ($2.66 \times 10^4 \text{ mol}^{-1} \text{ cm}^{-1}$) and enzyme activity was expressed as nanokats per milligram of total proteins.

Quantification of H₂O₂ by Xylenol Orange Assay

H₂O₂ production in wild-type and *CaPO2*-OX Arabidopsis plants was monitored using xylenol orange assay (Gay et al., 1999; Bindschedler et al., 2001, 2006). The xylenol orange reagent was freshly prepared and remained stable for 6 to 8 h. One milliliter of solution [25 mM FeSO₄ and 25 mM (NH₄)₂SO₄, dissolved in 2.5 M H₂SO₄] was added to 100 mL of 125 µM xylenol orange and 100 mM sorbitol. Arabidopsis leaves were infiltrated with 10⁷ cfu mL⁻¹ of *Pst* DC3000 supplemented with either 1 mM KCN or 2.5 µM DPI. Fifteen leaf discs (0.28 cm²) excised using a cork borer were floated on 1 mL distilled water. To measure the H₂O₂ amount, 120 µL medium was withdrawn at various time points, briefly centrifuged for 15 s at 13,000g, and 100 µL of the supernatant was immediately added to 1 mL of xylenol orange reagent. The reaction mixture was incubated for 30 min at room temperature before measuring the A_{560} against the blank containing 100 µL distilled water. H₂O₂ production was expressed as relative values with 100% corresponding to the maximum value of the elicited samples.

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers DQ489711 (*CaPO2*), AF053343 (*CaBPR1*), AF244122 (*CaPR4*), AF244121 (*CaPR10*), AF442387 (*CaPOA1*), AF442388 (*CaDEF1*), AY262059 (*CaOSM1*), AF313766 (*CaSAR82*), At1g64280 (*NPR1*), At2g14610 (*PR1*), At1g75040 (*PR5*), At2g29450 (*GST1*), and At4g05320 (*UBQ*).

Supplemental Data

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

Supplemental Figure S1. Amino acid sequence alignment of pepper peroxidase *CaPO2*.

Supplemental Figure S2. Generation of the *CaPO2*-OX Arabidopsis mutants that expressed the *CaPO2* gene.

Supplemental Figure S3. Quantification of H₂O₂ in leaves of wild-type (Col-0) and *CaPO2*-OX lines of Arabidopsis, using the xylenol orange assay.

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LITERATURE CITED

- Alvarez ME, Pennell RI, Meijer PJ, Ishikawa A, Dixon RA, Lamb C (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* 92: 773–784
- Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 55: 373–399
- Arnhold J (2004) Free radicals-friends or foes? Properties, functions, and secretion of human myeloperoxidase. *Biochemistry (Mosc)* 69: 4–9
- Babior BM (1984) The respiratory burst of phagocytes. *J Clin Invest* 73: 599–601

- Baulcombe DC (1999) Fast forward genetics based on virus-induced gene silencing. *Curr Opin Plant Biol* 2: 109–113
- Bindschedler LV, Dewdney J, Blee KA, Stone JM, Asai T, Plotnikov J, Denoux C, Hayes T, Gerrish C, Davies DR, et al (2006) Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. *Plant J* 47: 851–863
- Bindschedler LV, Minibayeva F, Gardber SL, Gerrish C, Davies DR, Bolwell GP (2001) Early signaling events in the apoplastic oxidative burst in suspension cultured French bean cells involve cAMP and Ca^{2+} . *New Phytol* 151: 185–194
- Bolwell GP, Blee KA, Butt VS, Davies DR, Gardner SL, Gerrish C, Minibayeva F, Rowntree EG, Wojtaszek P (1999) Recent advances in understanding the origin of the cell wall oxidative burst in plant cells. *Free Radic Res* 31: S137–S145
- Bolwell GP, Davies DR, Gerrish C, Auh CK, Murphy TM (1998) Comparative biochemistry of the oxidative burst produced by rose and French bean cells reveals two distinct mechanisms. *Plant Physiol* 116: 1379–1385
- Bolwell GP, Wojtaszek P (1997) Mechanisms for the generation of reactive oxygen species in plant defence—a broad perspective. *Physiol Mol Plant Pathol* 51: 347–366
- Bradley DJ, Kjellbon P, Lamb CJ (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell* 70: 21–30
- Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159
- Clough SH, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16: 735–743
- Chung E, Seong E, Kim YC, Chung EJ, Oh SK, Lee S, Park JM, Joung YH, Choi D (2004) A method of high frequency virus-induced gene silencing in chili pepper (*Capsicum annuum* L. cv. Bukang). *Mol Cell* 17: 377–380
- De Biasi MG, Astolfi S, Acampora A, Zuchi S, Fonzo V, Santangelo E, Caccia R, Badiani M, Soressi GP (2003) A H_2O_2 -forming peroxidase rather than a NAD(P)H-dependent O_2^- synthase may be the major player in cell death responses controlled by the *Pto-Fen* complex following fenthion treatment. *Funct Plant Biol* 30: 409–417
- Desikan R, Burnett EC, Hancock JT, Neill SJ (1998) Harpin and hydrogen peroxide induce the expression of a homologue of gp91^{phox} in *Arabidopsis thaliana* suspension cultures. *J Exp Bot* 49: 1767–1771
- Do HM, Hong JK, Jung HW, Kim SH, Ham JH, Hwang BK (2003) Expression of peroxidase-like genes, H_2O_2 production, and peroxidase activity during the hypersensitive response to *Xanthomonas campestris* pv. *vesicatoria* in *Capsicum annuum*. *Mol Plant Microbe Interact* 16: 196–205
- Doke N (1983) Involvement of superoxide anion generation in hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal cell wall components. *Physiol Plant Pathol* 23: 345–357
- Gay C, Collins J, Gebicki JM (1999) Hydroperoxide assay with the ferric-xylenol orange complex. *Anal Biochem* 273: 149–155
- Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J (2000) The *RPM1* plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for oxidative burst and hypersensitive cell death. *Plant J* 23: 441–450
- Groom OJ, Torres MA, Fordham-Skelton P, Hammond-Kosak KE, Robison NJ, Jones JDG (1996) *RbohA*, a rice homologue of mammalian pg91^{phox} respiratory burst oxidase gene. *Plant J* 10: 515–522
- Hammerschmidt R, Nuckles EM, Kuć J (1982) Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Physiol Plant Pathol* 20: 73–82
- Hammond-Kosack KE, Jones JDG (1996) Resistance gene-dependent plant defense responses. *Plant Cell* 8: 1773–1791
- Hilaire E, Young SA, Willard LH, McGee JD, Sweat T, Chittoor JM, Guikema JA, Leach JE (2001) Vascular defense responses in rice: peroxidase accumulation in xylem parenchyma cells and xylem wall thickening. *Mol Plant Microbe Interact* 14: 1411–1419
- Hiraga S, Sasaki K, Ito H, Ohashi Y, Matsui H (2001) A large family of class III plant peroxidases. *Plant Cell Physiol* 42: 462–468
- Jabs T (1999) Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. *Biochem Pharmacol* 57: 231–245
- Jones JDG, Dangl JL (2006) The plant immune system. *Nature* 444: 323–329
- Jung HW, Hwang BK (2000) Isolation, partial sequencing, and expression of pathogenesis-related cDNA genes from pepper leaves infected by *Xanthomonas campestris* pv. *vesicatoria*. *Mol Plant Microbe Interact* 13: 136–142
- Kawano T (2003) Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. *Plant Cell Rep* 21: 829–837
- Keller T, Damude HG, Werner D, Doerner P, Dixon RA, Lamb C (1998) A plant homologue of the neutrophil NADPH-oxidase gp91^{phox} subunit gene encodes a plasma membrane protein with Ca^{2+} binding motifs. *Plant Cell* 10: 255–266
- Kim YJ, Hwang BK (2000) Pepper gene encoding a basic pathogenesis-related 1 protein is pathogen and ethylene inducible. *Physiol Plant* 108: 51–60
- Kim YJ, Martin GB (2004) Molecular mechanisms involved in bacterial speck disease resistance of tomato. *Plant Pathol J* 20: 7–12
- Koch E, Slusarenko A (1990) Arabidopsis is susceptible to infection by downy mildew fungus. *Plant Cell* 2: 437–445
- Laloi C, Apel K, Danon A (2004) Reactive oxygen signaling: the latest news. *Curr Opin Plant Biol* 7: 323–328
- Lamb CJ, Dixon RA (1997) The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol Plant Mol Biol* 48: 251–275
- Lee SC, Hwang BK (2005) Induction of some defense-related genes and oxidative burst is required for the establishment of systemic acquired resistance in *Capsicum annuum*. *Planta* 221: 790–800
- Levine A, Tenhaken R, Dixon RA, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79: 583–593
- Liu Y, Schiff M, Dinesh-Kumar SP (2002) Virus-induced gene silencing in tomato. *Plant J* 31: 777–786
- Lorrain S, Vailleau F, Balagué C, Roby D (2003) Lesion mimic mutants: key for deciphering cell death and defense pathways in plants? *Trends Plant Sci* 8: 263–271
- Love AJ, Yun BW, Laval V, Loake GJ, Milner JL (2005) *Cauliflower mosaic virus*, a compatible pathogen of Arabidopsis, engages three distinct defense-signaling pathways and activates rapid systemic generation of reactive oxygen species. *Plant Physiol* 139: 935–948
- Mackey D, Belkadir Y, Alonso JM, Ecker JR, Dangl JL (2003) *Arabidopsis* RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell* 112: 379–389
- Mehdy MC (1994) Active oxygen species in plant defense against pathogens. *Plant Physiol* 105: 467–472
- Muraglia I, Tarantino D, Vannini C, Bracale M, Carravieri S, Soave C (2004) *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to paraquat-induced photooxidative stress and to nitric oxide-induced cell death. *Plant J* 38: 940–953
- Orozco-Cárdenas ML, Narváez-Vásquez J, Ryan CA (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. *Plant Cell* 13: 179–191
- Park HJ, Doke N (2005) Convenient assay of O_2^- generated on potato tuber tissue slices treated with fungal elicitor by electron spin resonance—no secondary oxidative burst induction by H_2O_2 treatment. *Plant Pathol J* 21: 283–287
- Park JM (2005) The hypersensitive response, a cell death during disease resistance. *Plant Pathol J* 21: 99–101
- Passardi F, Cosio C, Penel C, Dunand C (2005) Peroxidases have more functions than a Swiss army knife. *Plant Cell Rep* 24: 255–265
- Passardi F, Penel C, Dunand C (2004) Performing the paradoxical: how plant peroxidase modify the cell wall. *Trends Plant Sci* 9: 534–540
- Pellinen R, Palva T, Kangasjärvi J (1999) Subcellular localization of ozone-induced hydrogen peroxide production in birch (*Betula pendula*) leaf cells. *Plant J* 20: 349–356
- Romero-Puertas MC, Rodríguez-Serrano M, Corpas FJ, Gómez M, del Río LA, Sandalio LM (2004) Cadmium-induced subcellular accumulation of O_2^- and H_2O_2 in pea leaves. *Plant Cell Environ* 27: 1122–1134
- Tenhaken R, Levine A, Brisson LE, Dixon RA, Lamb C (1995) Function of the oxidative burst in hypersensitive disease resistance. *Proc Natl Acad Sci USA* 92: 4158–4163
- Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB (1997) Subcellular localization of H_2O_2 in plants: H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J* 11: 1187–1194

- Torres MA, Dangel JL** (2005) Functions of respiratory burst oxidase in biotic interactions, abiotic stress and development. *Curr Opin Plant Biol* **8**: 397–403
- Torres MA, Dangel JL, Jones JDG** (2002) *Arabidopsis* gp91^{phox} homologues, *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc Natl Acad Sci USA* **99**: 517–522
- Torres MA, Jones JDG, Dangel JL** (2005) Pathogen-induced, NADPH oxidase-derived reactive oxygen intermediates suppress spread of cell death in *Arabidopsis thaliana*. *Nat Genet* **37**: 1130–1134
- Torres MA, Ouchi H, Hamada S, Machida C, Hammond-Kosak KE, Jones JDG** (1998) Six *Arabidopsis thaliana* homologues of the human respiratory burst oxidase (gp91^{phox}). *Plant J* **14**: 365–370
- Wagner D, Przybyla D, Op den Camp R, Kim C, Landgraf F, Lee KP, Wüsch M, Laloi C, Nater M, Hideg E, et al** (2004) The genetic basis of singlet oxygen-induced stress responses of *Arabidopsis thaliana*. *Science* **306**: 1183–1185
- Yoshioka H, Numata N, Nakajima K, Katou S, Kawakita K, Rowland O, Jones JDG, Doke N** (2003) *Nicotiana benthamiana* gp91^{phox} homologs *NbrbohA* and *NbrbohB* participate in H₂O₂ accumulation and resistance to *Phytophthora infestans*. *Plant Cell* **15**: 706–718
- Yun SC, Kim BS, Cha AR, Pack JP** (2006) Ozone: changing anthracnose (caused by *Colletotrichum acutatum*) severity and accelerating hypersensitive response in pepper. *Plant Pathol J* **22**: 271–277
- Zhang X, Zhang L, Dong F, Gao J, Galbraith DW, Song CP** (2001) Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *Plant Physiol* **126**: 1438–1448
- Zimmerlin A, Wojtaszek P, Bolwell GP** (1994) Synthesis of dehydrogenation polymers of ferulic acid with high specificity by a purified cell-wall peroxidase from French bean (*Phaseolus vulgaris* L.). *Biochem J* **299**: 747–753