Hydrogen Peroxide Acts as a Second Messenger for the Induction of Defense Genes in Tomato Plants in Response to Wounding, Systemin, and Methyl Jasmonate

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The systemic accumulation of both hydrogen peroxide (H$_2$O$_2$) and proteinase inhibitor proteins in tomato leaves in response to wounding was inhibited by the NADPH oxidase inhibitors diphenylene iodonium (DPI), imidazole, and pyridine. The expression of several defense genes in response to wounding, systemin, oligosaccharides, and methyl jasmonate also was inhibited by DPI. These genes, including those of four proteinase inhibitors and polyphenol oxidase, are expressed within 4 to 12 hr after wounding. However, DPI did not inhibit the wound-inducible expression of genes encoding prosystemin, lipoxygenase, and allene oxide synthase, which are associated with the octadecanoid signaling pathway and are expressed 0.5 to 2 hr after wounding. Accordingly, treatment of plants with the H$_2$O$_2$-generating enzyme glucose oxidase plus glucose resulted in the induction of only the later-expressed defensive genes and not the early-expressed signaling-related genes. H$_2$O$_2$ was cytochemically detected in the cell walls of vascular parenchyma cells and spongy mesophyll cells within 4 hr after wounding of wild-type tomato leaves, but not earlier. The cumulative results suggest that active oxygen species are generated near cell walls of vascular bundle cells by oligogalacturonide fragments produced by wound-inducible polygalacturonase and that the resulting H$_2$O$_2$ acts as a second messenger for the activation of defense genes in mesophyll cells. These data provide a rationale for the sequential, coordinated, and functional roles of systemin, jasmonic acid, oligogalacturonides, and H$_2$O$_2$ signals for systemic signaling in tomato plants in response to wounding.

INTRODUCTION

Reactive oxygen species (ROS) are common components of the defense responses of plants against pathogen and herbivore attacks. Inoculation of plant tissues with pathogens or treatment of cell cultures with microbial elicitors causes an oxidative burst characterized by the rapid generation of hydrogen peroxide (H$_2$O$_2$; reviewed by Low and Merida, 1996; Lamb and Dixon, 1997; Bolwell, 1999). Similarly, ROS are generated in plant tissues in response to wounding (Angelini et al., 1990; Bradley et al., 1992; Olson and Varner, 1993; Felton et al., 1994; Bi and Felton, 1995; Orozco-Cárdenas and Ryan, 1999). Mechanical stimulation of isolated cells (Yahraus et al., 1995; Gus-Mayer et al., 1998) and the treatment of cell suspension cultures with plant cell wall–derived oligogalacturonic acid (OGA; Legendre et al., 1993; Stennis et al., 1998) also generate H$_2$O$_2$ accumulation. Wound-induced H$_2$O$_2$ accumulation is observed both locally and systemically in leaves of several plant species, apparently caused by OGA that was released by a systemically wound-induced polygalacturonase (PG; Bergey et al., 1999; Orozco-Cárdenas and Ryan, 1999).

H$_2$O$_2$ can act as a local signal for hypersensitive cell death and also as a diffusible signal for the induction of defensive genes in adjacent cells (Alvarez et al., 1998). For example, transgenic potato plants that overexpress a fungal glucose oxidase gene and protein contain a constitutive increase in sublethal levels of H$_2$O$_2$ and exhibit enhanced disease resistance (Wu et al., 1995, 1997). Similarly, transgenic tobacco plants engineered to have low levels of antioxidant defenses (enzymes) show higher constitutive and inducible levels of H$_2$O$_2$ and pathogenesis-related (PR) proteins, together with increased sensitivity and general resistance to pathogen challenge (Chamongpol et al., 1998; Mittler et al., 1999).

In several model systems investigated in plants, the oxidative burst and the accumulation of H$_2$O$_2$ appear to be mediated by the activation of a membrane-bound NADPH oxidase complex (Doke et al., 1996; Lamb and Dixon, 1997; Ogawa et al., 1997; Del Rio et al., 1998; Potikha et al., 1999; Pei et al., 2000). In animal cells, this enzymatic complex consists of two membrane-associated polypeptides (gp91-phox and gp22-phox) that become active when at least three proteins from the cytosol (p47-phox, p67-fox, and rac) bind to the membrane components (Jones, 1994; Henderson and...
Plant homologs of the animal NADPH oxidase protein subunits have been identified (Desikan et al., 1996; Xing et al., 1997; Potikha et al., 1999), and some of their genes have been sequenced (Groom et al., 1996; Keller et al., 1998; Torres et al., 1998). How the plant NADPH oxidase is regulated is still unknown. Some chemical inhibitors of the NADPH oxidase enzyme complex found in mammalian neutrophils inhibit the pathogen-, elicitor-, and wound-induced accumulation of \( \text{H}_2\text{O}_2 \) derived from the oxidative burst in plants (Levine et al., 1994; Auh and Murphy, 1995; Alvarez et al., 1998; Piedras et al., 1998; Orozco-Cárdenas and Ryan, 1999). In mammalian cells, the induction of defense genes by \( \text{H}_2\text{O}_2 \) involves the activation of the NF-\( \kappa \)B transcription factor by mediating its release from the inhibitory I\( \kappa \)B proteins (Li and Karin, 1999). In plants, the mechanisms by which \( \text{H}_2\text{O}_2 \) activates genes are not understood.

A model has been presented (Farmer and Ryan, 1992) for the expression of defense-related genes in tomato leaves in response to wounding and systemin. In this model, systemin initiates a cascade of intracellular events resulting in the activation of a cytoplasmic phospholipase that releases linolenic acid from membranes. Linolenic acid is converted to jasmonic acid, which is a powerful activator of genes coding for both signal pathway enzymes and defensive proteinase inhibitors and polyphenol oxidase. The model was modified recently to note that signal pathway genes are expressed within 0.5 hr after wounding, whereas defensive genes are expressed within 4 hr (Ryan, 2000). Recently, PG was shown to be among the early-expressed genes (Bergey et al., 1999), which raised questions concerning its role in the signal transduction pathway, because it was known to produce oligogalacturonide fragments from plant cell walls that are activators of both the defensive genes (Ryan and Farmer, 1991) and of the production of \( \text{H}_2\text{O}_2 \) (Stennis et al., 1998) in tomato leaves.

In this study, \( \text{H}_2\text{O}_2 \) was generated in vascular bundles of tomato leaves in response to wounding and behaved as if it was a diffusible signal for the expression of defense genes in mesophyll cells but not for signaling pathway genes in vascular bundle cells. Based on these results, a modified signal transduction pathway model is proposed (Figure 1) for the wound- and elicitor-induced defense response in which \( \text{H}_2\text{O}_2 \) is generated by wound-inducible PG and acts as a second messenger for the induction of defensive genes in mesophyll cells.

**RESULTS**

The localized and systemic activation of a leaf PG gene and its enzyme activity occur in response to wounding of the leaves of several plant families (Bergey et al., 1999). The time course of induction of PG activity in leaves correlates well with the accumulation of ROS, namely \( \text{H}_2\text{O}_2 \) (Orozco-Cárdenas and Ryan, 1999). Because oligogalacturonide fragments produced by PG are known to induce a substantial oxidative burst and the activation of defense gene expression (Legendre et al., 1993; Stennis et al., 1998), a relationship between the wound-inducible \( \text{H}_2\text{O}_2 \) and the wound-inducible defense gene activation after PG induction was investigated (Orozco-Cárdenas and Ryan, 1999).

A membrane-bound NADPH oxidase has been implicated in the production of ROS during the defense response of plants against pathogen and herbivore attacks (Doke et al., 1996; Low and Merida, 1996; Lamb and Dixon, 1997; Orozco-Cárdenas and Ryan, 1999). Accordingly, some known chemical inhibitors of the mammalian neutrophil NADPH oxidase inhibit the generation of ROS after pathogen infection in plants (Levine et al., 1994; Auh and Murphy, 1995; Piedras et al., 1998) and the accumulation of \( \text{H}_2\text{O}_2 \) in wounded or systemin-treated tomato leaves (Orozco-Cárdenas and Ryan, 1999). The production of \( \text{H}_2\text{O}_2 \) from ROS in tomato leaves likely results from the action of the enzyme SOD (Auh and Murphy, 1995). Therefore, the effect of three different NADPH oxidase inhibitors on the accumulation of proteinase inhibitor I and II proteins in leaves of young tomato plants in response to wounding were investi-
gated. As shown in Figure 2, when young, excised tomato plants were supplied through their cut stems for 30 min with solutions of the inhibitors diphenylene iodonium (DPI), pyridine, or imidazole, the accumulation of proteinase inhibitors I and II in response to wounding was severely diminished compared with untreated controls. Among the three inhibitors, DPI had the greatest inhibitory effect. Figure 3 shows that increasing concentrations of DPI (10 to 250 μM) progressively inhibited the accumulation of wound-inducible proteinase inhibitors in a concentration-dependent manner. The concentration of DPI required for half-maximal inhibition was ~50 μM (Figure 3).

DPI also inhibited the accumulation of proteinase inhibitors induced in tomato plants after treatment with different chemical elicitors of the wound response (Figure 4). DPI-pretreated plants accumulated significantly lower levels of proteinase inhibitor I in their leaves than control plants in response to systemin, OGA, chitosan, and the plant hormone methyl jasmonate (Figure 4). Together, these results suggest that an active NADPH oxidase enzyme is necessary for defense gene activation in response to wounding and chemical elicitors and the generation of H₂O₂ and may be involved as a signaling pathway component.

Glucose oxidase has been used to generate H₂O₂ in plants (Wu et al., 1995, 1997; Alvarez et al., 1998). Supplying plants with glucose oxidase together with glucose causes a continuous production of H₂O₂ within plant tissues (Levine et al., 1994; Alvarez et al., 1998). Excised tomato plants were incubated in a buffer solution containing glucose, gluconate, glucose oxidase, or a mixture of both glucose and glucose oxidase, and the accumulation of proteinase inhibitors I and II was assayed. Treatment with either compound or with the enzyme alone caused a slight to moderate increase of both proteinase inhibitor proteins I and II over levels observed in untreated controls (Figure 5). However, when the plants were supplied with glucose plus glucose oxidase, the levels of inhibitors I and II were induced to accumulate to ~80% of the levels found in wounded plants (Figure 5). This result suggests that the H₂O₂ generated by glucose oxidase within the apoplast was able to trigger signaling events leading to the induction of proteinase inhibitor protein accumulation.

To further investigate the role of H₂O₂ in defense gene activation, the effect of DPI on the wound induction of defense genes at the mRNA level was investigated. Gel blot analyses of total RNA isolated from wounded leaves of plants that had been preincubated in buffer alone (untreated controls) or pretreated with DPI were performed using cDNA clones of different wound-inducible genes as probes. These cDNAs encoded for two groups of functionally related proteins (Ryan, 2000). The first group included the signaling pathway-associated proteins prosystemin, lipoxygenase, and allene

**Figure 2.** Inhibition of Wound-Inducible Accumulation of Proteinase Inhibitors I and II Proteins by Different Chemical Inhibitors of NADPH Oxidase.

Fourteen-day-old tomato plants were excised at the base of the stems and supplied with solutions of phosphate buffer alone (Control and Wound), 40 mM pyridine, 20 mM imidazole, or 100 μM DPI in phosphate buffer for 30 min. Plants, except controls, were wounded and incubated in water under light as described in Methods. Proteinase inhibitors I (Inh I) and II (Inh II) were assayed immunologically in leaf juice 24 hr later. Data are means ±SD; n = 6.

**Figure 3.** Inhibition of Wound-Inducible Accumulation of Proteinase Inhibitors by the NADPH Oxidase Inhibitor DPI at Different Concentrations.

Plants were treated and assayed as described for Figure 1. Inh I, proteinase inhibitor I; Inh II, proteinase inhibitor II. Data are means ±SD; n = 6.
oxide synthase (AOS). The second group included the defense-related proteinase inhibitors I and II, cathepsin D inhibitor, carboxypeptidase inhibitor, and polyphenol oxidase. The mRNA corresponding to the signaling pathway–related genes, which were first expressed 0.5 to 2 hr after wounding and maximized at 4 hr, were not affected by DPI (Figure 6). On the other hand, the RNA encoding defense genes, which were first expressed ~4 hr after wounding and maximized at 8 to 12 hr, were inhibited by DPI. Therefore, DPI appeared to specifically block the wound-induced expression of defensive genes but not the activation of signaling genes. Interestingly, the wound-induced expression of the gene encoding the tomato leaf PG occurs early along with that of signaling genes, and the synthesis of its mRNA was not affected by DPI. This finding suggests that this cell wall pectin-degrading enzyme has a signaling role by generating oligouronides, which are known chemical elicitors of the oxidative burst (Legendre et al., 1993; Stennis et al., 1998; Lee et al., 1999). The resulting H$_2$O$_2$ appears to act as a second messenger for the expression of defensive genes, but not for the signal pathway genes. In an earlier study of the effects of systemin on the oxidative burst in tomato suspension cultured cells, Stennis et al. (1998) found that when systemin alone was added to the cells, it did not cause an oxidative burst. Within a few hours after addition, however, systemin did potentiate a severalfold increase of the oxidative burst caused by OGA. This indicates that the components required to produce the oxidative burst by OGA were induced by systemin. The components were not identified, and NADPH oxidase activity was not measured in these cells.

The expression of defensive and signaling genes was investigated after the treatment of excised tomato plants with the glucose oxidase–glucose H$_2$O$_2$-generating system. The plants were assayed for mRNAs for both the genes associated with signaling and those associated with defense. In leaves of plants treated with both glucose and glucose oxidase, the five genes involved in plant defense were induced, with an activation kinetics similar to that observed after wounding (Figure 7). However, none of the signaling genes was induced after the glucose oxidase treatment (Figure 7). Glucose, glucose oxidase, and the gluconate product alone (data not shown) did not cause the accumulation of detectable levels of defense or signaling gene mRNAs. Together, the analyses of wound-inducible defense gene expression indicate that H$_2$O$_2$ plays a key role as a second messenger in the induction of the defense genes but not in the regulation of the signaling genes.

The cytochemical dye 3,3-diaminobenzidine (DAB) visually localizes wound-inducible H$_2$O$_2$ throughout the leaves of wounded tomato plants (Orozco-Cárdenas and Ryan, 1999). H$_2$O$_2$ accumulates mainly around the wound sites and

![Figure 4. Inhibition of Elicitor-Induced Accumulation of Inhibitor I by DPI.](image1)

Plants were supplied with either phosphate buffer alone or 100 μM DPI in phosphate buffer for 30 min and transferred for 30 min to the same buffer solution containing 25 nM systemin, 250 μg/mL OGA, or 125 μg/mL chitosan or exposed to methyl jasmonate (MeJ) vapor as described in Methods. After each treatment, plants were incubated in water for 24 hr and then immunologically assayed for proteinase inhibitor I content in leaf juice. Data are means ± SD; n = 6.

![Figure 5. H$_2$O$_2$-Mediated Accumulation of Proteinase Inhibitors I and II Proteins in Tomato Leaves.](image2)

Fourteen-day-old tomato plants were excised and incubated in phosphate buffer alone (Control and Wound) or in buffer containing 50 μM glucose (Glu), 2.5 units/mL glucose oxidase (Oxidase), glucose plus glucose oxidase (Glu + Oxidase), or 50 μM gluconate (GlcO) for 2 hr. Thereafter, plants were incubated in water for 24 hr and assayed immunologically for proteinase inhibitors I (Inh I) and II (Inh II) content in leaf juice. Data are means ± SD; n = 6.
within the major and minor veins of the leaves, reaching levels of ~1 to 10 μM. DAB staining is blocked by pretreatment of the plants with different NADPH oxidase inhibitors and by catalase treatment after wounding of the leaves (M.L. Orozco-Cárdenas and C.A. Ryan, unpublished results). To further investigate the subcellular location of H$_2$O$_2$ generation and/or accumulation in the wounded leaves, we used a cerium perhydroxide (CeCl$_3$)-based cytochemical technique (Bestwick et al., 1997). In leaves of unwounded young tomato plants, CeCl$_3$ deposits, indicative of the presence of H$_2$O$_2$, were found in developing lignified secondary cell walls of xylem vessels (Figure 8A) but were not observed in the cell walls of vascular parenchyma (Figure 8B) or mesophyll cells (Figure 9A). On the other hand, in leaves of wounded plants, electron-dense CeCl$_3$ deposits were detected in the cell walls of vascular parenchyma (Figures 8C and 8D) and in nearby spongy mesophyll cells in both wounded and unwounded (systemic) leaves (Figures 9B to 9D). Similar deposits were found in cell walls of leaves of transgenic tomato plants overexpressing prosystemin (Figure 9E). Heavy CeCl$_3$ staining often was observed in the cell walls of spongy mesophyll cells facing intercellular spaces, a few cells away from the vascular traces (Figures 9C and 9D). The cytosol and internal cell organelles, including the chloroplasts, mitochondria, peroxisomes, nuclei, and tonoplasts of these cells, were almost completely free of electron-dense material. However, CeCl$_3$ deposits were observed readily within the chloroplasts of palisade and spongy mesophyll cells of plant leaves pretreated with paraquat, a ROS-inducing herbicide (data not shown), indicating that CeCl$_3$ could penetrate throughout the leaves and permeate cell membranes, as has been reported (Pellinen et al., 1999). In addition, supplying excised tomato plants with the DPI inhibitor before wounding did not result in an accumulation of CeCl$_3$ deposits in the cell walls of the vascular parenchyma and the mesophyll cells of the leaves (Figure 9F).

**DISCUSSION**

ROS are generated in plant tissues and organs during plant growth and development and also in response to environmental and biological stress (reviewed by Dangl et al., 1996; Greenberg, 1996; Pennell and Lamb, 1997). A previous study using several known chemical inhibitors of NADPH oxidase implicated this enzyme complex in the generation of ROS and the accumulation of H$_2$O$_2$ in wounded and unwounded leaves of several plant species in response to mechanical wounding (Orozco-Cárdenas and Ryan, 1999). The accumulation of H$_2$O$_2$ occurs near wound sites and also in distal unwounded leaves, indicating that the process is regulated by a systemic signaling system. In the present study, NADPH oxidase inhibitors were used to investigate their effects on the wound induction of defense proteinase inhibitor proteins in leaves of tomato plants. All of the NADPH oxidase inhibitors tested had an inhibitory effect not only on the generation of H$_2$O$_2$ but on the accumulation of proteinase inhibitors I and II as well (Figures 2 and 3). This finding supported the hypothesis that the H$_2$O$_2$ produced by the NADPH oxidase in response to wounding may have a regulatory role in the wound inducibility of defensive proteins.

DPI, a suicide inhibitor that binds irreversibly to the flavonoid group of the membrane-associated gp91-phox subunit of the NADPH oxidase complex (O’Donnell et al., 1993), was the most effective of all of the inhibitors tested. As with wounding, DPI inhibited the accumulation of proteinase inhibitor I that was induced after treatment of the plants with chemical elicitors of the wound response, namely systemin, OGA, and chitosan, as well as methyl jasmonate (Figure 4).
Although high concentrations of DPI can affect other enzymes potentially involved in the generation of ROS, including extracellular peroxidases and nitric oxide (NO) synthase (Bolwell, 1999), the similar results obtained with different specific inhibitors strongly suggested that the wound-inducible accumulation of H$_2$O$_2$ was the result of the activation of the NADPH oxidase in the leaves. In addition, pretreatment of tomato plants with the NO synthase inhibitors 1,3-phenyltetrazolium1-oxyl-3-oxide did not affect the accumulation of the inhibitor proteins after wounding (data not shown).

Additional evidence that the ROS produced by the NADPH oxidase has a regulatory role in the induction of the defense genes was obtained from experiments with the H$_2$O$_2$-generating system previously was shown to cause the constitutive accumulation of sublethal levels of H$_2$O$_2$ in the apoplast of plant tissues and in the medium of suspension cell cultures, with the concomitant activation of a defense response equivalent to systemic acquired resistance (SAR). This treatment caused the plants to be more resistant to pathogen attacks (Wu et al., 1995, 1997; Alvarez et al., 1998).

Analysis of defense gene expression by using gel blots showed that DPI selectively inhibited the wound induction of defense genes involved directly in deterring pest attacks (i.e., proteinase inhibitors and polyphenol oxidase), but it did not inhibit the expression of genes that are part of the wound-signaling pathway (Figure 6). Glucose/glucose oxidase treatment of the young excised tomato plants induced the expression of only the defense genes that were inhibited by DPI and not the transcription of the signaling genes (Figure 7). By comparing the timing of the wound-inducible expression of the genes that have a direct role in plant defense and those of the signal transduction pathway, it is clear that the two classes of wound-inducible genes are differentially regulated in a temporal manner, as reported previously (Ryan, 2000).

H$_2$O$_2$ is known to directly regulate the expression of numerous genes, some of which are involved in plant defense and the hypersensitive response (Levine et al., 1994; Korsmeyer et al., 1995; Alvarez et al., 1998; Desikan et al., 2000; Kovtun et al., 2000). However, it also has been reported that H$_2$O$_2$ alone is not the only agent involved in the hypersensitive response or programmed cell death and that salicylic acid (SA) and NO both play important roles in the onset of pathogen-induced programmed cell death in plants and the establishment of SAR (Draper, 1997; Shirasu et al., 1997; Delledonne et al., 1998; Durner et al., 1998; Dorey et al., 1999). As discussed above, NO does not appear to be involved in the wound-inducible defense response. Furthermore, because SA-inducible PR genes such as $PR-1$ (Malamy and Klessig, 1992) were not found in wounded tomato leaves (data not shown), the accumulation of H$_2$O$_2$ in leaves in response to wounding probably does not directly involve SA or NO.

Histochemical techniques have been used for the detection of H$_2$O$_2$ generated in plant tissues during plant–pathogen interactions (Bestwick et al., 1997; Thordal-Christensen et al., 1997) and in response to abiotic stress (Pellinen et al., 1999). In lettuce leaves inoculated with the bacterial pathogen $Pseudomonas syringae pv phaseolicola$, Bestwick et al. (1997) reported the appearance of CeCl$_3$ deposits of variable intensity in the cell walls of spongy mesophyll cells facing intercellular spaces adjacent to infecting bacteria. In the wounded and unwounded leaves of tomato plants, scat-
tered and localized dense deposits of CeCl₃ were observed in the walls of vascular parenchyma cells (Figures 8C and 8D) and nearby spongy mesophyll cells (Figures 9B to 9E). The intensity of the CeCl₃ staining would correspond to sub-lethal levels of H₂O₂, not high enough to cause cell collapse and death, as observed by Bestwick et al. (1997). As noted above, using the DAB histochemical dye to detect H₂O₂ in wounded tomato leaves, we calculated the levels of wound-inducible H₂O₂ to be ~1 to 10 μM (Orozco-Cárdenas and Ryan, 1999), which again might not be high enough to cause hypersensitive cell death. In fact, no signs of necrotic tissue were observed in the transgenic tomato plants over-expressing prosystemin (McGurl et al., 1994), which constitutively accumulate H₂O₂ in their leaves (Figure 9E; Orozco-Cárdenas and Ryan, 1999).

The greatest accumulation of H₂O₂ was observed in the walls of spongy mesophyll cells facing large intercellular spaces (Figures 9C and 9D). These gas-filled cavities, which in some leaves accounted for as much as 70% of the leaf volume, constituted a labyrinth that surrounds the cells and

Figure 8. Cytochemical Localization of Wound-Inducible H₂O₂ in Vascular Bundles of Tomato Leaves.

(A) Electron-dense deposits of CeCl₃ indicative of the presence of H₂O₂ in developing secondary cell walls of xylem vessels (XV) of control unwounded leaves (arrows show typical deposits). Note that the cell walls of an adjacent vascular parenchyma cell (VP) show little CeCl₃ staining.

(B) Absence of CeCl₃ staining in the cell walls of vascular parenchyma (VP) and neighboring spongy mesophyll (SM) cells associated with the phloem in control unwounded leaves.

(C) H₂O₂ generation in the vascular bundle of a wounded tomato leaf 4 hr after wounding. H₂O₂ accumulates strongly in the cell walls of vascular parenchyma cells bordering spongy mesophyll cells and at the intercellular spaces (IS).

(D) Systemic accumulation of H₂O₂ in vascular bundles of upper unwounded leaves of young tomato plants 4 hr after wounding of the lower leaf. CC, companion cell; CW, cell wall; SE, sieve element. Bar = 5 μm.
Figure 9. Cytochemical Localization of Wound-Inducible H$_2$O$_2$ in Mesophyll Cells of Tomato Leaves.
is continuous with the spaces under stomata. The cell walls bordering these spaces were quite thin and were evolved to allow gas exchange (i.e., O₂ and CO₂) and substantial water loss through transpiration. The water and salts that make up the transpiration stream come from the xylem elements of the vascular traces, usually only a few cells away from any mesophyll element. If H₂O₂ is generated or overproduced in response to wounding in the cell walls of the vascular bundle cells, it can be readily transported in water through the apoplast and diffuse initially into the cells adjacent to each vein. The accumulation of H₂O₂ at high levels in cell walls adjacent to intercellular spaces in the spongy mesophyll can be explained by the rapid evaporation of water in these air-filled spaces, together with the lower ROS-scavenging activity at these sites. This also might represent a defense strategy for the plant, because stomata and the intercellular spaces are paths for invasion by microbial pathogens (Bestwick et al., 1997). In a recent report, H₂O₂ generated after treatment of tomato leaf epidermis with the oligosaccharide elicitors from plant (OGA) and fungal (chitosan) cell walls are shown to cause the closure of stomata (Lee et al., 1999), which can limit the entrance of pathogens into the plant. Stomata closure also is mediated by H₂O₂ through the activation of calcium channels (Pei et al., 2000).

Low-frequency systemic secondary oxidative bursts and hypersensitive cell death associated with the establishment of SAR have been observed in distant tissues and uninoculated leaves of Arabidopsis plants challenged with an avirulent pathogen (Alvarez et al., 1998). Interestingly, the primary and secondary oxidative bursts, which in this incompatible plant–pathogen interaction leads to hypersensitive cell death, were observed primarily adjacent to the veins, in so-called periveinal cells. These periveinal cells die more rapidly than other cells in the inoculated tissues and before the appearance of the hypersensitive response lesion (Alvarez et al., 1998). The rapid death of periveinal cells also has been observed in tomato leaves inoculated with race-specific elicitors from the fungus Cladosporium (Hammond-Kosack et al., 1994). The occurrence of specialized ROS-generating cells associated with differentiated vascular tissues also is suggested by the observations that the induction of defense-related responses and the hypersensitive cell death are developmentally regulated in tomato plants and cell cultures (Hammond-Kosack et al., 1994; Honne et al., 1998). Together, these observations suggest that the vascular bundles must play an important role in the generation of signals, such as H₂O₂, that regulate the defense response of plants against pathogens and herbivores.

The model shown in Figure 1 is consistent with what is known at present of both the signals and the signal transduction pathway enzymes that regulate systemic wound induction of signaling and defensive genes in tomato plants (Farmer and Ryan, 1992; Ryan, 2000). Upon wounding of the leaves by herbivores, systemin is released into the vascular system, where it activates the biosynthesis of jasmonic acid in vascular parenchyma cells, upregulating the synthesis of signal pathway genes, including PG. The gene products include prosystemin and some jasmonic acid biosynthetic enzymes, including isoforms of lipoxygenase and AOS, which are known to be synthesized in the vascular bundle cells (Jacinto et al., 1997; Kubigtsteltig et al., 1999). PG is likely to be found in the cell walls of the vascular bundle, where H₂O₂ is localized (Figure 7). The production of OGA by PG results in the synthesis of H₂O₂, which then diffuses out of the vascular bundles to mesophyll cells, where it activates the expression of genes coding for proteinase inhibitors and polyphenol oxidase. Previous ultrastructural studies of tomato leaves have shown that proteinase inhibitors synthesized in response to wounding are sequestered in the central vacuoles of mesophyll cells (Shumway et al., 1976; Narváez-Vásquez et al., 1993).

Interestingly, OGA initially was found to signal the induction of proteinase inhibitors several years ago (Ryan, 1988). Because the OGAs were not mobile, they were not considered further as systemic signals. The recent discovery of wound-inducible H₂O₂ in plant leaves and its relationships with OGA and defense gene activation has brought a new perspective and a more rational explanation of the temporal, spatial, and functional relationships among systemic wound signals. These include the polypeptide signal systemin, the

Figure 9. (continued).

(A) Spongy mesophyll cells of a control leaf from an unwounded plant do not exhibit H₂O₂ in the cell walls or intercellular spaces (IS).
(B) Accumulation of H₂O₂ in the bordering cell walls of a vascular parenchyma and a spongy mesophyll cell of a wounded leaf 4 hr after wounding.
(C) Accumulation of H₂O₂ in the cell walls of two spongy mesophyll cells facing an intercellular space 4 hr after wounding of the leaf.
(D) Systemic H₂O₂ accumulation in the cell walls of two spongy mesophyll cells facing an intercellular space 4 hr after wounding of the lower leaf.
(E) Constitutive accumulation of H₂O₂ in the cell wall of a spongy mesophyll cell of a leaf from a transgenic tomato plant overexpressing prosystemin. The proteinaceous material (P) within the central vacuole corresponds to aggregates containing proteinase inhibitor proteins that constitutively accumulate in transgenic tissue.
(F) Inhibition of wound-induced H₂O₂ accumulation by DPI. Leaf samples were obtained 4 hr after wounding.

C, chloroplast; CW, cell wall; M, mitochondrion; N, nucleus; P, protein aggregates of defensive inhibitor proteins; V, central vacuole. Bar in (A), (C), (D), and (F) = 2 μm; bar in (B) and (E) = 1 μm.
lipid signal jasmonic acid, the oligosaccharide signal OGA, and the inorganic chemical signal H₂O₂.

METHODS

Plant Growth

Tomato plants (Lycopersicon esculentum cv Castlemart) were grown from seed for 2 weeks in a growth chamber with 18-hr days (light at 300 μE·m⁻²·sec⁻¹) at 28°C and 6-hr nights at 18°C. Transgenic tomato plants expressing a prosystemin cDNA gene under the control of the cauliflower mosaic virus 3SS promoter (McGurl et al., 1994) were grown under the same conditions.

Assays

To assay chemical elicitors of the wound response, plants with two expanding leaves and a small terminal leaf were used. The plants were excised at the base of the stem with a razor blade, and the cut stem was placed in 10⁻³ M potassium phosphate buffer, pH 6.0, or in a solution of systemin (25 nM), oligogalacturonic acid (OGA; 0.5 mg/mL), or chitosan (125 μL), or a solution of systemin (25 nM), oligogalacturonic acid (OGA; 0.5 mg/mL) in phosphate buffer for 30 min. After treatment, the plants were incubated in water under constant light at 28°C. Total RNA pellets were dissolved in 25 mM Tris, 1 mM EDTA, pH 7.4, 5 mM Denhardt’s solution [1 × Denhardt’s solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA], 1% SDS, and 10% dextran sulfate). Radioactive 3²P-dCTP-labeled probes were generated by random priming according to the manufacturer's recommendations (DECA prime II kit; Ambion, Austin, TX) by using tomato cDNAs encoding allene oxide synthase (AOS) (Howe et al., 2000), lipoxigenase (Heitz et al., 1997), prosystemin (McGurl et al., 1992), leaf polygalacturonase catalytic subunit (Bergey et al., 1999), serine proteinase inhibitor I (Graham et al., 1985a), serine proteinase inhibitor II (Graham et al., 1985b), cathepsin D inhibitor (Hildemann et al., 1992), carboxypeptidase inhibitor (Moura and Ryan, 2000), and polyphenol oxidase (Constabel et al., 1995). An 18S rRNA gene probe was used as a loading control. Synthetic oligonucleotide probes were purified using Bio-spin P6 chromatography columns (Bio-Rad). The probes were heat denatured, added to the hybridization buffer, and incubated with the blocked membranes overnight at 65°C. Membranes were washed once with 2 × SSPE for 20 min at room temperature, twice with 2 × SSPE and 1% SDS for 15 to 30 min at 65°C, and then exposed for 15 to 32 hr to x-ray film or to a PhosphorImager (Bio-Rad).

Cytochemical Detection of H₂O₂

H₂O₂ was visualized at the subcellular level using CeCl₃ for localization (Bestwick et al., 1997; Pellinen et al., 1999). Electron-dense CeCl₃ deposits are formed in the presence of H₂O₂ and are visible by transmission electron microscopy. Briefly, tissue pieces (2 × 5 mm²) were excised from leaves of wounded and unwounded wild-type and transgenic tomato plants and then vacuum infiltrated with freshly prepared 5 mM CeCl₃ in 50 mM 3-(N-morpholino)-propanesulfonic acid at pH 7.2 for 30 min. Tissues then were fixed in 1.25% (v/v) glutaraldehyde and 1.25% (v/v) paraformaldehyde in 50 mM sodium cacodylate buffer (CAB), pH 7.2, for 1 hr at room temperature and kept overnight at 4°C. After fixation, tissues were washed twice for 10 min in CAB and postfixed for 45 min in 1% (v/v) osmium tetroxide in CAB. Tissues were then washed twice for 10 min in CAB and dehydrated in a graded acetone series (30, 50, 70, 80, 90, and 100% [v/v]) and critical point dried before embedding in Eponate 12 resin before staining with toluidine blue and examined using a Reichert Ultracut E Microtome (Leica AG, Wein, Austria) using a diamond knife (Delaware Diamond Knives, Wilmington, DE), mounted on nickel grids (200 mesh), and examined without staining with a transmission electron microscope (model JEM-1200Ex; JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 80 kV.

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