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Activation of Host Defense Mechanisms by Elevated Production of H_2O_2 in Transgenic Plants

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Active oxygen species have been postulated to perform multiple functions in plant defense, but their exact role in plant resistance to diseases is not fully understood. We have recently demonstrated H_2O_2-mediated disease resistance in transgenic potato (*Solanum tuberosum*) plants expressing a foreign gene encoding glucose oxidase. In this study we provide further evidence that the H_2O_2-mediated disease resistance in potato is effective against a broad range of plant pathogens. We have investigated mechanisms underlying the H_2O_2-mediated disease resistance in transgenic potato plants. The constitutively elevated levels of H_2O_2 induced the accumulation of total salicylic acid severalfold in the leaf tissue of transgenic plants, although no significant change was detected in the level of free salicylic acid. The mRNAs of two defense-related genes encoding the anionic peroxidase and acidic chitinase were also induced. In addition, an increased accumulation of several isoforms of extracellular peroxidase, including a newly induced one, was observed. This was accompanied by a significant increase in the lignin content of stem and root tissues of the transgenic plants. The results suggest that constitutively elevated sublethal levels of H_2O_2 are sufficient to activate an array of host defense mechanisms, and these defense mechanisms may be a major contributing factor to the H_2O_2-mediated disease resistance in transgenic plants.

Plants have evolved sophisticated biochemical mechanisms to exert self-defense against pathogen infections. Upon specific recognition of pathogens, plants respond by activating a battery of defense reactions. Examples of the defense reactions include the formation of antimicrobial phytoalexins (Hahlbrock and Scheel, 1989; Dixon and Harrison, 1990), the fortification of cell wall structure (Barber et al., 1989; Bradley et al., 1992; Brisson et al., 1994), the induction of hydrolytic enzymes and other defense-related proteins (Bowles, 1990; Dixon and Lamb, 1990), and hypersensitive cell death (Klement, 1982; Lamb et al., 1989). Such a wide array of defense responses is brought about by specific interactions between elicitor(s) originated from the pathogen and receptor(s) of the host cell (Atkinson, 1993). However, the intracellular signal transduction pathways that lead to the activation of the whole plant defense cascade are still not fully understood.

One of the most peculiar events in the early phase of plant-pathogen interactions is the rapid and transient production of AOS by the plant, namely the oxidative burst (Mehdy, 1994; Baker and Orlandi, 1995; Low and Merida, 1996). This response has been observed in numerous plant-pathogen systems involving fungi (Doke, 1983; Vera-Estrella et al., 1992), bacteria (Adam et al., 1989; Keppler et al., 1989), viruses (Doke and Ohashi, 1988), or elicitors (Apostol et al., 1989; Baker et al., 1993). The oxidative burst has also been correlated with the occurrence of a hypersensitive response (Tenhaken et al., 1995). The generation of AOS in incompatible interactions appears to be a biphasic process. The initial increase of AOS is rapid and nonspecific and is seen in compatible interactions as well. However, a second strong and prolonged oxidative burst occurs only when plant cells are in contact with pathogens or their elicitors that are capable of triggering hypersensitive cell death (Baker and Orlandi, 1995; Low and Merida, 1996). The predominant forms of AOS detected in plant-pathogen interactions include O_2^-, H_2O_2, and $OH^\cdot$. Although the enzymatic components responsible for the formation of AOS have not been biochemically characterized, the involvement of an oxidase analogous to the multisubunit NADPH oxidase in mammalian phagocytes (Morel et al., 1991; Segal and Abo, 1993) has been suggested to catalyze the production of O_2^- (Vianello and Macri, 1991; Auh and Murphy, 1995).

There is ample evidence indicating that AOS, and H_2O_2 in particular, generated in the oxidative burst, perform multiple important functions in early defense responses of the plant. H_2O_2 has been shown to inhibit the growth and viability of diverse microbial pathogens (Peng and Kuc, 1992; Kiraly et al., 1993; Wu et al., 1995), which may directly suppress attempted invasion by the pathogens. The oxidative potential of H_2O_2 also contributes to plant cell wall strengthening during plant-pathogen interactions through the peroxidase-mediated cross-linking of Pro-rich structural proteins (Bradley et al., 1992; Brisson et al., 1994). In addition, activation of phytoalexin biosynthesis by H_2O_2 produced during the oxidative burst has been observed in

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Abbreviations: AOS, active oxygen species; GO, Glc oxidase (EC 1.1.3.4); PR, pathogenesis-related; SA, salicylic acid.

suspension cells (Apostol et al., 1989; Degousée et al., 1994) and in potato (*Solanum tuberosum*) tuber tissues (Chai and Doke, 1987). Moreover, H_2O_2 has been implicated to play a role not only in triggering hypersensitive cell death but also in limiting the spread of cell death by induction of cell protectant genes in surrounding cells (Levine et al., 1994; Tenhaken et al., 1995).

On the other hand, the role of H_2O_2 in the downstream intracellular signaling leading to the expression of PR proteins and the induction of systemic resistance is still rather controversial. Chen et al. (1993) reported that SA, the key signal molecule required for the induction of systemic acquired resistance, inhibited tobacco catalase. Treatment of tobacco leaves with exogenous H_2O_2 induced expression of the *PR-1* gene. Hence, it was suggested that inhibition of catalase by SA results in the accumulation of H_2O_2, which in turn can induce PR proteins and systemic resistance (Chen et al., 1995). In support of this model, Conrath et al. (1995) found that 2,6-dichloroisonicotinic acid, a synthetic inducer of systemic acquired resistance, and other biologically active analogs also inhibit catalase in vivo. Furthermore, SA and 2,6-dichloroisonicotinic acid also inhibit the other major H_2O_2-scavenging ascorbate peroxidase enzyme. However, Bi et al. (1995) and Neuenschwander et al. (1995) found no significant change in either catalase activity or H_2O_2 level in the pathogen-challenged or systemic tissues of tobacco following infection. These authors argued that H_2O_2 was unlikely to be the secondary messenger in the signaling of systemic acquired resistance.

We have recently obtained transgenic potato plants that express a fungal *GO* gene and accumulate GO protein in the extracellular space. GO catalyzes the oxidation of β-D-Glc by molecular O_2; the reaction generates the AOS H_2O_2. These plants contained constitutively elevated levels of H_2O_2 and exhibited enhanced disease resistance (Wu et al., 1995). The transgenic plants were further characterized as part of this study in an attempt to elucidate the mechanisms of disease resistance and to gain insight into the functions of H_2O_2 in the induction of disease resistance. Here we show evidence that constitutively elevated sublethal levels of H_2O_2 are sufficient for triggering an array of plant defense responses and such host defense activation appears to be the primary mechanism of H_2O_2-mediated disease resistance in transgenic plants expressing *GO*.

MATERIALS AND METHODS

Plant Material and Plant Growth Conditions

Transgenic lines of potato (*Solanum tuberosum* cv Russet Burbank) transformed with the *GO* gene under the control of a figwort mosaic virus 34S promoter were described previously (Wu et al., 1995). All nontransgenic and transgenic plants were vegetatively propagated through cuttings and maintained in growth chambers with a 16-h photoperiod of 60 to 70 $\mu E\ m^{-2}\ s^{-1}$, at 21 and 16°C for day and night, respectively. Potato lines used in this study were 25587–3, -9, -12, -26, and -43. As previously reported for line 25587–3 (Wu et al., 1995), these transgenic lines accumulated GO protein in the apoplast and had increased levels of H_2O_2 in the leaf tissue. Tissues were collected from plants for SA quantification, RNA and protein analyses, and lignin content determination at times as indicated.

Disease Resistance Tests

All transgenic and nontransgenic potato plants were transferred from tissue culture into greenhouse potting mix when they were approximately 5 to 8 cm tall. For all tests the potting mixture consisted of 50% Metro-Mix 200 and 12% Redi-Earth (both from Grace-Sierra Horticultural Products [Milpitas, CA], with 25% coarse sand and 12% silty clay loam soil [all v/v]). Plants for the *Alternaria solani* early blight test were transferred to 10-cm^2 pots and maintained at 21°C, 75% RH, and 12 h of light until use. Plants for the *Verticillium dahliae* wilt test were transplanted as described below during initiation of the assay.

Inoculum of *V. dahliae* was prepared by harvesting spores from 5- to 8-d-old streak cultures growing in the dark at 20°C on potato-dextrose agar (Difco, Detroit, MI). Conidia were collected by flooding the plates with sterile, distilled water and adjusting the concentration to 10^7/mL. As the potato plants were removed from tissue culture the roots were dipped into the conidial suspension and the plants were immediately transplanted one per pot into moist potting mixture in 15-cm round pots. An additional 5 mL of conidial suspension was pipetted onto the soil at the base of each plant. Four plants of each line were inoculated. The plants were then maintained in a growth chamber at 20°C and 75% RH; initially the plants were kept for 24 h in the dark, but then they were exposed to a normal 12-h light/dark cycle. Water was applied as needed to keep the soil moist. Beginning 26 d after planting, the disease severity (percentage of foliar chlorosis and wilt) was recorded at various intervals until completion of the test.

Inoculum of *A. solani* was prepared by adding sterile distilled water to 10-d-old cultures growing at 25°C and 12 h of light on vegetable juice agar (163 mL of V-8 Juice [Campbell Soup, Camden, NJ], 837 mL of distilled water, 3 g of $CaCO_3$, and 15 g of Difco Bacto-Agar). Conidia were dislodged in the water, the suspension was filtered through two layers of cheesecloth, and the concentration was adjusted to 4.4×10^4/mL. When the potted potato plants were 10 to 14 cm tall and had at least eight well-developed leaves, they were inoculated by spraying all leaves with the conidial suspension until they were thoroughly wet. Four plants of each line were inoculated. The plants were then incubated in a large plastic humidity tent in the dark at 21°C with complete leaf wetness provided by intermittent misting. After 24 h a 12-h light cycle was resumed and the misting was continued. Leaf disease severity was evaluated 3 d after inoculation on both the upper and lower leaves of each plant.

Measurement of SA

Leaf tissue samples of 0.5 g fresh weight each were harvested from 8-week-old potato plants and frozen immediately in liquid nitrogen. Free and total SA (the sum of free and conjugated SA) was determined as previously de-

scribed by Enyedi and Raskin (1993). Corrections were made for all data by using SA-spiked samples, which gave estimated SA recovery ranging from 49 to 100%.

Northern and Western Analyses

Total RNA was isolated from leaf tissues of 7- to 8-week-old plants using the TRIzol Reagent and the procedure suggested by the manufacturer (Life Technologies). Twenty micrograms of total RNA per lane was loaded on 1 or 1.2% agarose gels containing 2.2 M formaldehyde and separated by electrophoresis at 8 V/cm for approximately 4 h. Ethidium bromide was included in loading buffer to ensure that equal amounts of all RNA samples were used for electrophoresis. RNAs were blotted by capillary transfer to Hybond-N^+ membranes (Amersham) in 20× SSC buffer, pH 7.0, and were fixed onto the membranes by baking under vacuum pressure at 80°C for 1 h (Sambrook et al., 1989). cDNA fragments were excised with appropriate restriction enzymes from the cDNA clones of a potato acidic chitinase (Kombrink et al., 1995) and a tomato anionic peroxidase (Roberts and Kolattukudy, 1989) and then labeled with [^{32}P]dCTP by the random primed method (Boehringer Mannheim). The RNA blots were pretreated with hybridization buffer (5× SSC, pH 7.0, containing 35% formamide, 5× Denhardt's solution, 1% SDS, and 300 μg/mL yeast tRNA) at 37°C for 2 h and hybridized in the same buffer to the labeled cDNA probes. Low-stringency washing (twice for 20 min at 37°C followed by twice for 20 min at 50°C in 2× SSC and 1% SDS) was used because of the nonhomologous origins of the cDNA probes. The hybridized RNA blots were autoradiographed using X-Omat x-ray films (Kodak).

For detection of anionic peroxidase protein, extracellular wash fluid was collected by vacuum infiltrating leaves with 20 mM phosphate buffer, pH 6.0, containing 5 mM EDTA, and then by centrifuging at 500*g* for 10 min. SDS-PAGE electrophoresis and western-blot analysis were performed as described previously (Wu et al., 1995) using 5 μg of protein of each sample and the antibody raised against a synthetic peptide (NH_2-FTGEQNSPPNANSARGYE-COOH) made according to the amino acid sequence of the tomato anionic peroxidase (E.B. Lawrence and D. M. Shah, unpublished data). The amount of protein on western blots was quantified by comparing it with standards using the IS-2000 digital imaging system (Alpha Innotech, San Leandro, CA).

Peroxidase Assay and Lignin Measurement

Extracellular wash fluid was collected from leaves of transgenic and control plants as described above. Total protein concentration was determined using Micro BCA Protein Assay reagents (Pierce). Five micrograms of extracellular protein was loaded on Vertical IEF SepraGels, pH 3.0–10.0 (Integrated Separation Systems, Natick, MA), and separated by electrophoresis for 75 min at 200 and 400 V, respectively. Peroxidase activity was detected in-gel by incubating the samples at room temperature for 20 min in 25 mM phosphate buffer, pH 7.0, containing 20% methanol, 5 mM EDTA, 100 mM H_2O_2, and 0.6 mg/mL 4-chloro-1-naphthol.

Lignin content in plant tissues was measured by the reaction with thioglycolic acid as described by Lange et al. (1995). Cell wall preparations were made from stem and root tissues of *GO*-transgenic and control plants. After derivatization with thioglycolic acid, the relative lignin contents in the tissue samples were compared spectrophotometrically by the A_{280}.

RESULTS

Broad-Spectrum Disease Resistance of *GO*-Transgenic Plants

GO-transgenic potato plants have been shown to have enhanced resistance to *Phytophthora* late blight and *Erwinia* soft rot (Wu et al., 1995). In this study the plants were tested for the resistance to *Verticillium* sp. wilt and *Alternaria* early blight. The percentage of wilt-severity ratings for each plant line infected with *V. dahliae* was plotted over time to construct disease progression curves (Fig. 1). Disease symptoms did not appear until about 26 d after planting, when the four *GO*-expressing lines and the control lines all started with low levels of disease. However, between 26 and 46 d after planting disease severity increased much faster in the control lines than in the *GO*-transgenic lines, which had 50 to 75% lower disease severity 46 d after planting (Fig. 2). By 69 d after planting, both the control and transgenic lines had similar high levels of disease. Expression of the *GO* gene in these test lines greatly delayed disease onset and slowed the rate of symptom development, both of which are common components of enhanced disease resistance. In a separate experiment, these

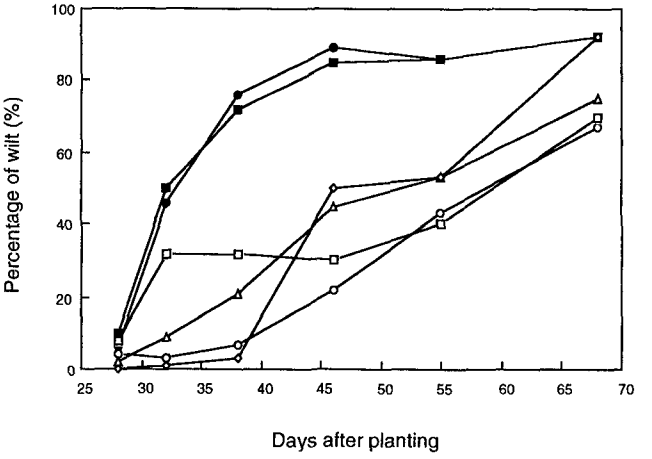

Figure 1. Percentage of wilt symptoms over time in *GO*-transgenic and control potato lines infected with *V. dahliae*. Transgenic and control potato plants were inoculated with *V. dahliae* by root dipping and base soil injection with spore suspension at the time of planting. Disease severity was recorded during the test as percentages of foliar chlorosis and wilt. Potato lines represented are untransformed cv Russet Burbank control (●), vector-transformed control (■), and four *GO*-expressing lines, 22587–9 (○), 22587–26 (□), 22587–30 (△), and 22587–43 (◇).

Figure 2. Disease symptoms caused by *V. dahliae* on potato plants of a *GO*-transgenic and the untransformed control line. Transgenic and control potato plants were inoculated with spores of *V. dahliae* as described in Figure 1. Plants were photographed 46 d after planting and inoculation. Top row, Four infected and one uninoculated cv Russet Burbank control plants (Rus. Bur.); bottom row, four infected and one uninoculated *GO*-transgenic plants (22587–43).

lines showed resistance to *Verticillium* wilt that was equal to or better than that of a commercial resistant variety of potato cv Russet Ranger (data not shown).

Foliar disease severity of *Alternaria* sp. early blight was evaluated 5 d after inoculation by visually estimating the percentage of the diseased area on the three or four upper and three or four lower leaves (Table I). Disease severity was greatest on the lower leaves, ranging from 67 to 75% in the cv Russet Burbank and vector-transformed control lines, but was only 25 to 42% in the *GO*-expressing lines. The disease severity was similarly reduced in the upper leaves with 28 to 33% severity in the control lines and only 4 to 10% severity in the *GO*-transgenic lines. These differences were sustained during the 7 d of the test, and they were statistically significant with an LSD (0.05) of 20 and 18% for the lower and upper leaves, respectively.

Induced Expression of Defense-Related Genes

The expression of genes encoding plant PR proteins has been closely correlated with the onset of systemic acquired resistance (Hunt and Ryals, 1996; Ryals et al., 1996). To determine whether expression of genes encoding PR proteins was activated in uninfected *GO*-transgenic plants containing elevated levels of H_2O_2, we analyzed the mRNA levels of an anionic peroxidase and an acidic chitinase by northern-blot hybridization. Because the DNA probes used originated from different plant species or cultivars, low-stringency conditions were used in the RNA blot analyses. As illustrated in Figure 3, the chitinase cDNA probe (Kombrink et al., 1995) hybridized to a 1.2-kb RNA, in addition to a 1.6-kb transcript and another larger species of unknown identity. The 1.6- and 1.2-kb transcripts corresponded to the mRNA of the constitutive and the pathogen-inducible forms of potato acidic chitinases, respectively (Ancillo et al., 1995). The expression of the inducible acidic chitinase in all *GO*-transgenic lines was clearly activated, since it was barely detectable in the untransformed cv Russet Burbank control line (Fig. 3A). As previously reported for line 25587–3 (Wu et al., 1995), the transgenic lines all had a 2- to 3-fold increase in H_2O_2 production in leaf tissue as the result of *GO* expression. Thus, the elevated levels of H_2O_2 in leaf tissue of the transgenic plants resulted in the induction of a PR gene encoding acidic chitinase.

Anionic peroxidases are a class of defense proteins in which the expression has been correlated with plant responses to pathogen infection (Kolattukudy et al., 1992). Northern-blot hybridization using the cDNA of anionic peroxidase from tomato as a probe (Roberts and Kolattukudy, 1989) detected different levels of RNA transcripts in *GO*-transgenic lines. Although all *GO*-transgenic lines showed higher levels of the peroxidase mRNA than the untransformed cv Russet Burbank control line, transgenic lines 22587–3, 22587–9, and 22587–12 gave stronger hybridization signals than lines 22587–26 and 22587–43 (Fig. 3B). To determine whether such increases at the transcriptional level also led to increased production of the anionic peroxidase protein, western-blot analysis was performed using the antibody raised against a synthetic peptide made

Table I. *Enhanced resistance of GO-transgenic potato plants to early blight disease caused by A. solani*

Plants were inoculated with a conidial suspension of *A. solani*. Surface areas covered by disease lesions on the upper and lower leaves of the inoculated plants were measured 5 d after inoculation. Potato line designation: Rus. Bur., Untransformed Russet Burbank potato; 17227-1, vector-transformed control line; 22587-9, 22587-26, 22587-30, and 22587-43, four *GO*-transgenic lines.

Potato Line	Percentage of Diseased Leaf Area	
	Upper leaves	Lower leaves
Rus. Bur.	28.3	66.7
17227-1	33.3	75.0
22587-9	7.0	25.0
22587-26	10.0	41.7
22587-30	5.3	25.0
22587-43	4.0	28.3
	$LSD_{0.05}$ = 18.2	$LSD_{0.05}$ = 19.9

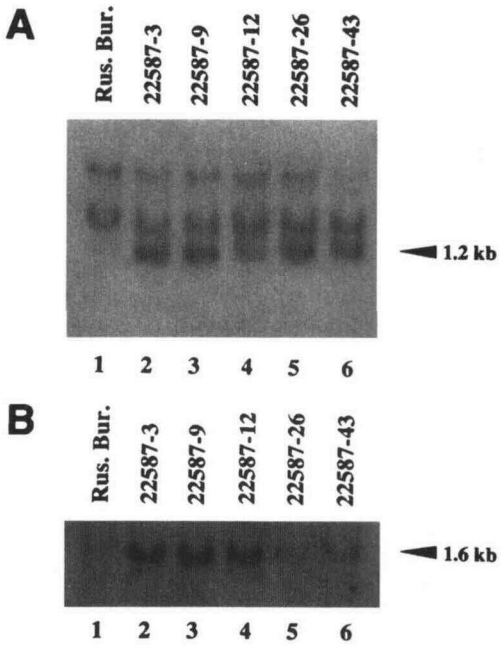

Figure 3. Northern-blot detection of expression of an acidic chitinase and an anionic peroxidase in *GO*-transgenic potato. Total RNA was isolated from leaf tissues of 7- to 8-week-old plants. After agarose gel separation and northern transfer, RNA blots were hybridized with ^{32}P-labeled cDNA probes of acidic chitinase (A) or anionic peroxidase (B). Rus. Bur., Untransformed cv Russet Burbank potato (lane 1); 22587–3 to 22587–43, five *GO*-transgenic lines (lanes 2–6).

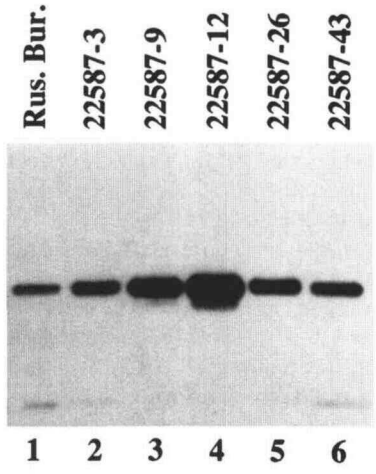

Figure 4. Immunoblot detection of extracellular accumulation of an anionic peroxidase in *GO*-transgenic potato. Extracellular wash fluid was collected from leaves of transgenic and control plants. Five micrograms of each protein sample was subjected to SDS-PAGE and western-blot immunodetection using the antibody raised against the tomato anionic peroxidase. A protein of approximately 40 kD was detected on the immunoblot. Rus. Bur., Untransformed cv Russet Burbank potato (lane 1); 22587–3 to 22587–43, five *GO*-transgenic lines (lanes 2–6).

according to the amino acid sequence of the tomato anionic peroxidase. Western-blot detection of the extracellular anionic peroxidase in the *GO*-transgenic potato plants revealed much higher increases than did the northern analysis. The transgenic lines had 6- to 20-fold increases in the accumulation of the peroxidase protein compared with the cv Russet Burbank control line (Fig. 4), whereas only 2- to 6-fold induction of the mRNA was observed by northern-blot analysis (Fig. 3B). It is known that potato contains a multigene family for anionic peroxidase (Roberts and Kolattukudy, 1989). Therefore, it is possible that the antibody used here detected more than one member of the gene family. Certain posttranscriptional or translational effects may also contribute to the high abundance of the peroxidase detected in *GO*-transgenic plants.

Increased Accumulation of Total SA in Transgenic Plants

SA accumulation has been shown to be a requirement for the induction of PR gene expression and the establishment of systemic acquired resistance in tobacco and Arabidopsis (Gaffney et al., 1993; Delaney et al., 1994). However, its involvement in induced resistance of potato has not been demonstrated. Because the PR gene expression was detected in *GO*-transgenic plants, we measured the levels of SA in leaf tissues of the plants. As shown in Figure 5, little change in levels of free SA was observed between the transgenic and the untransformed control lines. However, the levels of total SA in tissues of *GO*-transgenic lines were approximately 4- to 5-fold higher when compared with the cv Russet Burbank control line. The high levels of total SA in the transgenic plants indicate that there was an increased accumulation of conjugated SA.

Induction of Extracellular Peroxidases and Increase of Lignin Content

In transgenic potato plants, the GO protein was targeted into the apoplast (Wu et al., 1995). Therefore, production of

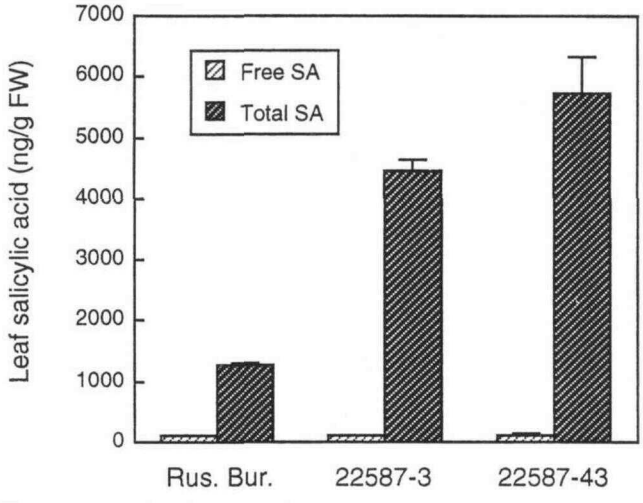

Figure 5. Levels of free and total SA in the leaf tissue of *GO*-transgenic potato plants. Leaf tissues were harvested from 8-week-old plants of *GO*-transgenic (22587–3 and 22587–43) and cv Russet Burbank control (Rus. Bur.) lines and analyzed for free and total SA content. The data represent the means ± SD of three replicates. FW, Fresh weight.

H_2O_2 by GO would occur primarily in the apoplastic space. It has been postulated that H_2O_2 generated on the cell surface is involved in the peroxidase-mediated lignification of plant cell walls, which can result in enhanced disease resistance in the plant (Matern and Kneusel, 1988; Vance et al., 1980). We examined changes in all extracellular peroxidases in the transgenic plants. The extracellular wash fluid was subjected to IEF gel electrophoresis, which was followed by an in-gel peroxidase activity assay. The assay detected at least three different isoforms of peroxidase in which the pI ranged from 3.25 to 4.0 (Fig. 6). *GO*-transgenic plants contained higher levels of these extracellular peroxidases than the untransformed cv Russet Burbank control plants. One peroxidase with a pI of 3.5 showed most evident induction in the *GO*-expressing transgenic lines (Fig. 6, arrowhead).

To determine whether the induction of extracellular peroxidases in *GO*-expressing plants induced lignification, the content of lignin in the cell walls of transgenic and control plants was compared. Table II shows the relative lignin contents in two transgenic plants and the control lines as determined by a thioglycolic acid assay using purified cell wall material from stem and root tissues. The amounts of lignin in the two transgenic lines, 22587–26 and 22587–43, were approximately 30 to 45% higher, respectively, than in the cv Russet Burbank control line. Such increases were found in both stem and root tissues.

Table II. *Increased lignin content in tissues of GO-transgenic potato plants*

Cell wall material was purified from stem and root tissues of 8-week-old potato plants. Lignin content was assayed by the reaction with thioglycolic acid and by measuring the A_{280}. Potato line designation: Rus. Bur., Untransformed or Russet Burbank potato; 22587-26 and 22587-43, two *GO*-transgenic lines.

Potato Line	Lignin Content	
	Stem tissue	Root tissue
	A_{280}/mg cell wall	
Rus. Bur.	0.3962 ± 0.0039	0.4764 ± 0.0005
22587-26	0.5476 ± 0.0124	0.6862 ± 0.0130
22587-43	0.5328 ± 0.0020	0.7016 ± 0.0034
	$LSD_{0.05}$ = 0.1076	$LSD_{0.05}$ = 0.1034

DISCUSSION

The transgenic plants that constitutively produce elevated sublethal levels of H_2O_2 provide a unique tool with which to study the physiological functions of H_2O_2, especially its role in plant pathogenesis and disease resistance. In this report we show evidence that increased production of H_2O_2 in planta activates a series of host defense mechanisms in the plant. The responses of transgenic plants to elevated apoplastic production of H_2O_2, which include the increase of total SA accumulation, the induction of defense-related proteins, and the peroxidase-mediated lignification of the cell wall, are summarized in Figure 7.

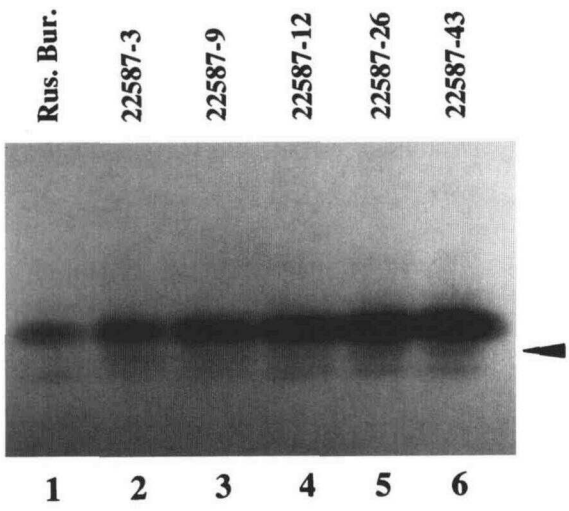

Figure 6. In-gel activity assay of extracellular peroxidases in *GO*-transgenic potato. Five micrograms of protein in extracellular wash fluid collected from leaves of transgenic and control plants was used for electrophoresis in an IEF gel. Peroxidase activity was detected by incubating the gel in a substrate buffer containing H_2O_2 and 4-chloro-1-naphthol. Rus. Bur., Untransformed cv Russet Burbank potato (lane 1); 22587–3 to 22587–43, five *GO*-transgenic lines (lanes 2–6).

We found that activation of multiple host defense mechanisms by elevated levels of H_2O_2 is likely involved in conferring broad-spectrum resistance to bacterial and fungal pathogens. The phytopathogens tested in this study and in a previous study (Wu et al., 1995) were not only diverse in pathogenicity but also different in their sensitivity to H_2O_2. For instance, *V. dahliae* was much less sensitive to inhibition by H_2O_2 than was *Erwinia carotovora* ssp. *carotovora* and *Phytophthora infestans* (Wu et al., 1995; G. Wu, and D.M. Shah, unpublished results). Thus, it is unlikely that the H_2O_2-mediated disease resistance is facilitated solely by the direct toxicity of H_2O_2 toward these pathogens. In fact, the levels of H_2O_2 in transgenic plants detected biochemically were less than 1 mmol/g fresh weight of leaf tissue (Wu et al., 1995). Such levels of H_2O_2 were significantly lower than the 10 mM required for in vitro inhibition of *V. dahliae* (data not shown). These results suggest that there is a common mechanism that leads to general and broad-spectrum disease resistance in *GO*-expressing plants, although direct antimicrobial effects of H_2O_2 may also contribute by suppressing pathogen growth.

Expression of PR proteins has been correlated with the induction of systemic acquired resistance in plants (Bol et al., 1990; Ryals et al., 1996). Some PR proteins, such as chitinases, β-1,3-glucanases, and thaumatin-like proteins, have been shown to have direct antifungal activities (Linthorst, 1991; Viggers et al., 1991; Woloshuk et al., 1991). The detection of a newly induced acidic chitinase mRNA in plants of *GO*-expressing lines, but not in the untransformed cv Russet Burbank control line (Fig. 3A), indicates that the transgenic plants were in the state of induced resistance. This conclusion was further supported by the finding that there were also increases of an anionic peroxidase at both transcriptional and posttranscriptional levels (Figs. 3B and 4). Like many other defense proteins, plant anionic peroxi-

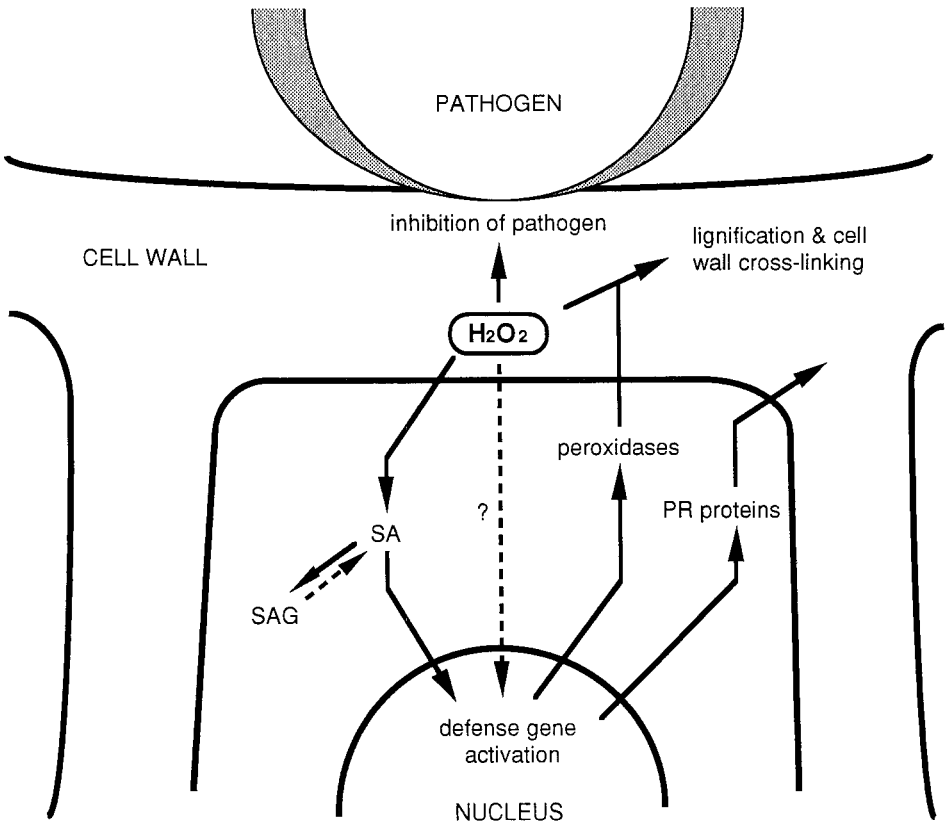

Figure 7. Hypothetical representation of host defense activation by extracellularly produced H_2O_2 in *GO*-transgenic plant. Apoplastically produced H_2O_2 inhibits the growth and viability of the invading pathogen. H_2O_2 also activates a number of host defense mechanisms. SA biosynthesis is elevated through stimulation of benzoic acid 2-hydroxylase activity. Conjugation of free SA leads to the accumulation of SA β-glucoside (SAG). The expression of defense-related proteins is induced by increased SA and perhaps also by H_2O_2 itself via a separate pathway. The increase and induction of extracellular peroxidases contribute to plant disease resistance through lignification and cell wall cross-linking. Ultimately, these responses result in enhanced and broad-spectrum disease resistance in the plant.

dases are induced by wounding, pathogen infection, or fungal elicitor treatment, and their role in plant defense against pathogen infection through suberization has been suggested (Kolattukudy et al., 1992).

Plants contain abundant isoenzymes of extracellular peroxidases that are postulated to play an integral role in the polymerization of cell wall components including lignin, suberin, and extensin (Gaspar et al., 1982). Like the anionic peroxidase noted above, other isoforms of cell wall peroxidases, such as lignin peroxidases, also perform defensive functions in the plant (Vance et al., 1980). Although the direct role of peroxidases in plant disease resistance has not been established, the association of increased peroxidase activity and the onset of systemic acquired resistance has been observed in a number of plant species, including cucumber, tobacco, and melons (Simons and Ross, 1970; Hammerschmidt et al., 1982; Smith and Hammerschmidt, 1990; Rasmussen et al., 1995). The induction of the acidic peroxidases in *GO*-transgenic plants (Fig. 6) may be one of the mechanisms that can detoxify elevated H_2O_2. As a result, these enzymes probably contribute to enhanced disease resistance. Consistent with this notion was the finding of increased lignin contents in tissues of the transgenic plants (Table II). Lignification upon pathogen challenge or wounding is believed to be an important active defense mechanism of higher plants. Its biological significance in disease resistance includes restricting diffusion of nutrients from the host to the pathogen and protecting cell wall structural components from degradation by enzymes produced by the pathogen (Ride, 1978). Therefore, extracellular peroxidase-mediated lignification found in *GO*-transgenic plants would conceivably result in suppression of pathogen ingress and thus would enhance plant disease resistance.

It was not surprising to find increased accumulation of total SA in tissues of *GO*-transgenic plants, because H_2O_2 was recently shown to stimulate SA biosynthesis in Arabidopsis and tobacco (León et al., 1995; Summermatter et al., 1995). H_2O_2 treatment of leaves resulted in accumulation of benzoic acid, the precursor of SA, as well as activation of benzoic acid 2-hydroxylase, the key enzyme for SA synthesis. However, unlike plants undergoing systemic acquired resistance that have elevated levels of both free and bound SA (Klessig and Malamy, 1994), we observed a significant increase of the conjugated SA only in the *GO*-expressing transgenic plants (Fig. 5). It is conceivable that SA pro-

duced in the plants was rapidly converted into nonactive conjugated forms. The conversion of free SA into conjugated forms is thought to be catalyzed by enzymes such as UDP-Glc:SA glucosyltransferase, which has been isolated from several plant species (Tanaka et al., 1990; Yalpani et al., 1992; Enyedi and Raskin, 1993). An increase in activity of similar enzymes in the transgenic potato plants probably was responsible for maintaining the relatively low levels of free SA.

Taken together, the results of the present study strongly suggest that H_2O_2 elevation is sufficient to activate host defense mechanisms and confer broad-spectrum disease resistance in transgenic potato plants. SA has been proposed to cause H_2O_2 elevation by inhibiting detoxifying enzymes such as catalase and ascorbate peroxidase (Chen et al., 1993; Conrath et al., 1995). Although the results of this work do not exclude this hypothesis, they show that H_2O_2 itself leads to SA accumulation in the leaf tissue. However, it remains unknown whether H_2O_2 exerts host defense activation only through SA or whether there is also an SA-independent pathway for induction of certain defense genes by H_2O_2 (Fig. 7). It is possible that, although SA was rapidly converted into conjugated forms, its production is sufficient to induce defense genes. On the other hand, there is evidence that certain defense genes of plants can be activated by H_2O_2 without SA. The observation that H_2O_2, but not SA, was a potent inducer of the *AoPR-1* gene implies the existence of a separate pathway for defense gene induction (Bi et al., 1995). It is interesting to note that the induction of a nopaline synthase promoter by H_2O_2 in transgenic tobacco plants is also independent of SA (Dai and An, 1995). Gene activation by H_2O_2 occurs in mammalian cells, in which H_2O_2 serves as a messenger mediating the release of the inhibitory subunit IκB from NF-κB, which results in the activation of the NF-κB transcription factor (Schreck et al., 1991).

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