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RESEARCH ARTICLE

Disease Resistance Conferred by Expression of a Gene Encoding H_2O_2-Generating Glucose Oxidase in Transgenic Potato Plants

Gusui Wu, Barry J. Shortt, Ellen B. Lawrence, Elaine B. Levine, Karen C. Fitzsimmons, and Dilip M. Shah[1]

Monsanto Company, 700 Chesterfield Village Parkway, St. Louis, Missouri 63198

Plant defense responses to pathogen infection involve the production of active oxygen species, including hydrogen peroxide (H_2O_2). We obtained transgenic potato plants expressing a fungal gene encoding glucose oxidase, which generates H_2O_2 when glucose is oxidized. H_2O_2 levels were elevated in both leaf and tuber tissues of these plants. Transgenic potato tubers exhibited strong resistance to a bacterial soft rot disease caused by *Erwinia carotovora* subsp *carotovora*, and disease resistance was sustained under both aerobic and anaerobic conditions of bacterial infection. This resistance to soft rot was apparently mediated by elevated levels of H_2O_2, because the resistance could be counteracted by exogenously added H_2O_2-degrading catalase. The transgenic plants with increased levels of H_2O_2 also exhibited enhanced resistance to potato late blight caused by *Phytophthora infestans*. The development of lesions resulting from infection by *P. infestans* was significantly delayed in leaves of these plants. Thus, the expression of an active oxygen species–generating enzyme in transgenic plants represents a novel approach for engineering broad-spectrum disease resistance in plants.

INTRODUCTION

Plants ward off pathogen infections by eliciting an array of defense mechanisms, including reinforcement of the cell wall (Kauss, 1987; Barber et al., 1989; Bradley et al., 1992), synthesis of phytoalexins and oxidation of phenolic compounds (Hahlbrock and Scheel, 1989; Dixon and Lamb, 1990), activation of defense-related genes (Bowles, 1990; Dixon and Harrison, 1990), and localization of cell death or the hypersensitive response (Keen, 1992). Associated with these reactions is a rapid and transient production of active oxygen species (AOS), such as the superoxide anion radical (O_2^-), hydroxyl radical ($OH^\cdot$), and hydrogen peroxide (H_2O_2). This process is referred to as an oxidative burst (Lamb et al., 1989; Mehdy, 1994). The accumulation of H_2O_2 and related AOS has been determined to be one of the earliest events that occurs at host–pathogen recognition; it has been postulated to play an important role in plant defense (Baker and Orlandi, 1995).

In addition to its oxidative potential in killing or inhibiting the growth of pathogens, H_2O_2 has been shown to be involved in a number of plant defense response processes. In plant cell suspension culture systems, H_2O_2 produced during the oxidative burst has been shown to activate the biosynthesis of phytoalexins (Apostol et al., 1989; Davis et al., 1993; Degousée et al., 1994) and to induce the cross-linking of the hydroxyproline-rich cell wall glycoproteins (Bradley et al., 1992; Brisson et al., 1994). It has been observed in several plant–pathogen systems that an incompatible interaction triggers the rapid second production of AOS following the first oxidative increase, but a compatible interaction (which leads to disease in the host) does not induce this second oxidative burst (Chai and Doke, 1987; Baker et al., 1991; Glazener et al., 1991). Recently, the H_2O_2 burst generated during incompatible interactions has been implicated not only as a local trigger for inducing hypersensitive cell death but also as a diffusible signal for activating cellular protectant genes, such as glutathione *S*-transferase, in surrounding cells (Levine et al., 1994). H_2O_2 may also be involved in the induction of systemic acquired resistance by salicylic acid (Chen et al., 1993). Salicylic acid inhibits catalase activity and thereby causes an increase in H_2O_2 levels; in turn, the elevated level of H_2O_2 induces the expression of pathogenesis-related proteins in plants (Klessig and Malamy, 1994).

Although H_2O_2 appears to play an important role in plant defense, no evidence directly links H_2O_2 generated in the plant with disease resistance. To increase H_2O_2 production in a plant and to study its effect on a plant's resistance to disease, we expressed the gene encoding glucose oxidase (GO) from *Aspergillus niger* in transgenic potato plants. GO (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) catalyzes the oxidation of β-D-glucose by molecular oxygen, yielding gluconic acid and H_2O_2. A number of bacteria and fungi produce GO, which has a putative antibiotic function, but GO has not been found in animals and plants (Frederick et al., 1990). Here we

[1] To whom correspondence should be addressed.

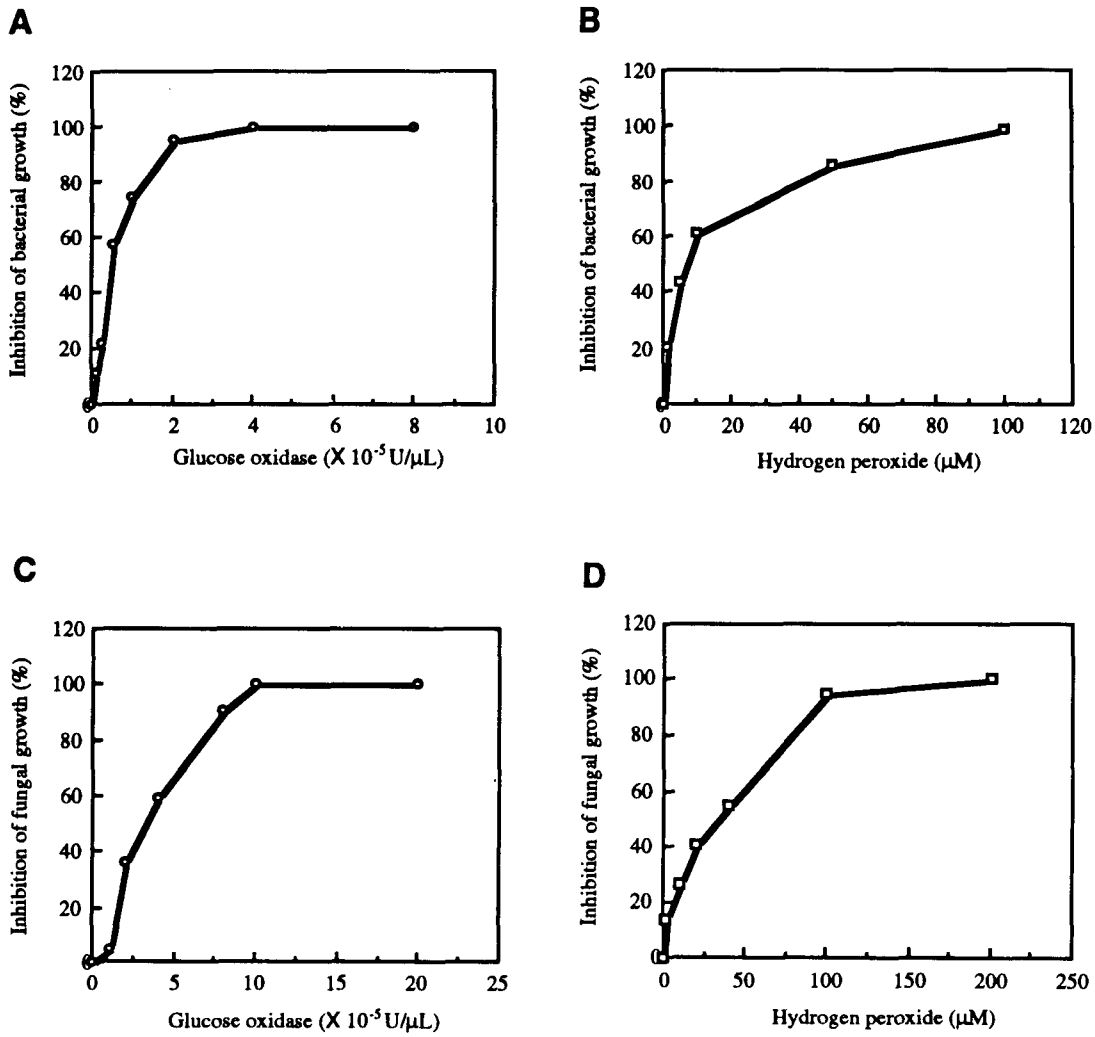

Figure 1. Growth Inhibition of *E. c. carotovora* and *P. infestans* by GO and H_2O_2.

(A) and **(B)** *E. c. carotovora* grown in potato dextrose medium.
(C) and **(D)** *P. infestans* cultured in M303 medium.
In **(A)** and **(C)**, various amounts of *A. niger* GO were added to the bacterial or pregerminated fungal cultures. In **(B)** and **(D)**, various amounts of H_2O_2 were added. Growth of the bacteria and the fungal hyphae was monitored by absorbency at 595 nm, and inhibition by GO or H_2O_2 was compared with water-treated controls. U, units.

show that expression of the fungal *GO* gene leads to elevated production of H_2O_2 in transgenic potato plants. The increased level of H_2O_2 in transgenic potato plants mediates strong resistance against soft rot caused by *Erwinia carotovora* subsp *carotovora* and enhanced resistance to late blight caused by *Phytophthora infestans*, two major diseases in potato that severely hinder the production, storage, transport, and marketing of potato worldwide (Pérombelon and Kelman, 1980; Robertson, 1991).

RESULTS

Growth Inhibition of *E. c. carotovora* and *P. infestans* by GO and H_2O_2

The H_2O_2-generating GO from *A. niger* was tested for its ability to inhibit the growth of *E. c. carotovora* and *P. infestans* in vitro. We observed, in the presence of glucose, significant

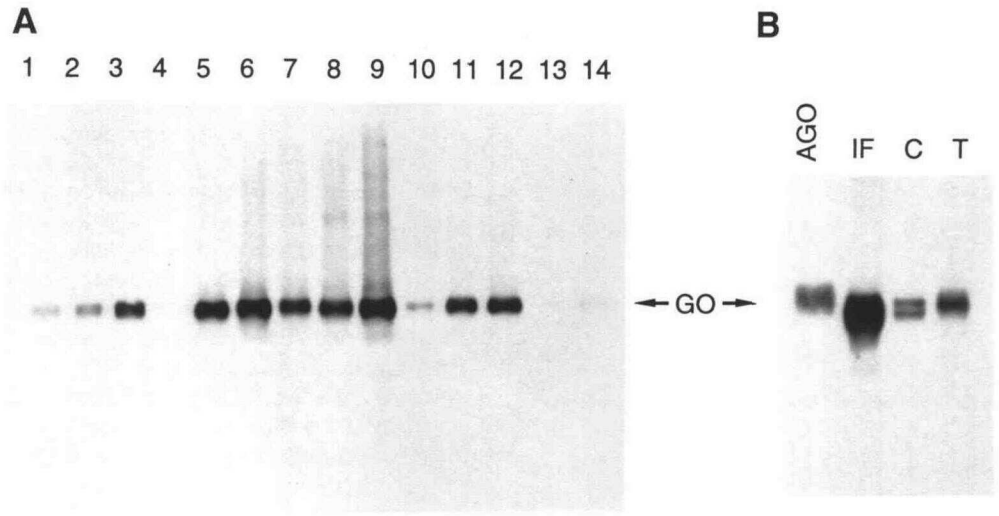

Figure 2. Accumulation and Apoplastic Localization of GO in Transgenic Potato Plants.

Extracts of potato leaf protein were subjected to SDS-PAGE and immunodetected using the antibody raised against *A. niger* GO (see Methods). **(A)** GO (~70 kD) detected in total leaf proteins of several transgenic potato lines. Lanes 1 to 3 contain 1, 2, and 5 ng of purified *A. niger* GO, respectively; lane 4, a vector-transformed control line; lanes 5 to 14, 10 independent transgenic potato lines expressing the *GO* gene at different levels. **(B)** Detection of GO in the intercellular fluid of transgenic potato plants. Leaves of transgenic line 22587-3 were infiltrated with 25 mM phosphate buffer, pH 7.0, containing 5 mM EDTA, and the intercellular fluid was collected. After the intercellular fluid was removed by centrifugation, leaf tissues were extracted to obtain the intracellular protein. Ten micrograms each of proteins from the intercellular fluid (IF) or from the intracellular fraction (C) and 20 μg of total leaf protein (T) were subjected to SDS-PAGE and detected by immunoblotting. The two protein bands detected in lane C may represent GO at different states of processing, including glycosylation and cleavage of the 22-amino acid signal peptide (Frederick et al., 1990). Five nonagrams of *A. niger* GO (AGO) was used as a marker.

inhibition of the growth of *E. c. carotovora* and *P. infestans* at very low concentrations of GO. Complete inhibition of the bacterial and fungal growth occurred when GO was added at concentrations of 4×10^{-5} and 10×10^{-5} units per μL of the assay mixture, respectively (Figures 1A and 1C). The in vitro inhibition of bacterial and fungal growth by GO was abolished in the presence of catalase (data not shown). H_2O_2 was also directly tested for its ability to inhibit the growth of *E. c. carotovora* and *P. infestans.* In the presence of 100 μM H_2O_2, *E. c. carotovora* and *P. infestans* exhibited little or no growth (Figures 1B and 1D), indicating high sensitivities of these pathogens to H_2O_2.

Gene Expression and Apoplastic Localization of GO in Transgenic Potato Plants

Potato (cv Russet Burbank) plants were transformed with an expression vector containing the *GO* gene controlled by the 35S promoter of figwort mosaic virus (Richins et al., 1987; Sanger et al., 1990). To determine the levels of GO produced in transgenic potato plants, immunoblot analysis was performed using the antibody raised against the fungal GO protein. More than 20 *GO*-expressing potato lines were obtained that had GO representing 0.02 to 0.45% of the total extractable leaf protein (Figure 2A). Enzymatic activity of GO in leaf extracts correlated positively with protein levels determined by immunoblot analysis (data not shown). Thus, enzymatically competent GO was produced in the transgenic plants.

The tubers from transgenic potato plants were also examined for expression of the *GO* gene. Although direct correlation was not observed between the relative GO level in leaves and in tubers for each potato line, GO was produced in tubers of several transgenic lines. Figure 3 shows immunoblot detection of GO in tubers of two transgenic potato lines, 22587-3 and 22587-34, that accumulated the protein at levels of 0.02 and 0.04% of the total extractable tuber protein, respectively. GO protein in the transgenic tubers was also enzymatically active (data not shown).

Because the chimeric *GO* gene introduced into potato plants contained its original 22-amino acid signal peptide sequence (Kriechbaum et al., 1989; Frederick et al., 1990), the subcellular targeting of GO in transgenic potato lines was examined. The GO protein was efficiently secreted into the culture medium when the transgene was transiently expressed in tobacco protoplasts (data not shown). Immunoblot analysis of the protein from the intercellular wash fluid of leaves showed that the majority of GO is secreted into the apoplast, although lower

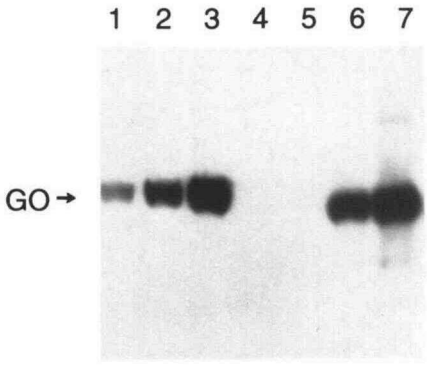

Figure 3. Expression of GO in Tuber Tissue of Two Transgenic Potato Lines.

Potato tuber proteins were extracted and GO was immunodetected as described in Figure 2. Lanes 1 to 3 contain 1, 2, and 5 ng of purified *A. niger* GO, respectively; lanes 4 to 7 were loaded with 20 μg of total tuber protein from untransformed Russet Burbank, vector-transformed control line 17227-1, and *GO*-transformed lines 22587-3 and 22587-34, respectively. GO at left indicates the position of glucose oxidase protein.

amounts of GO were also detected in the intracellular fractions (Figure 2B).

Elevation of H_2O_2 Levels in Transgenic Potato Plants

The levels of H_2O_2 in different tissues of transgenic potato plants were determined. The elevation of H_2O_2 levels in tissues of transgenic plants could be detected visually by placing young plants or plant tissues into a medium containing KI and starch (Olson and Varner, 1993). H_2O_2 diffusing from tissues of transgenic plants was indicated by the purple-blue color in surrounding areas in the medium. Using such an assay, elevated production of H_2O_2 was observed in leaf, tuber, and root tissues, whereas little or no H_2O_2 could be detected in the nontransgenic control plants (Figure 4A). Because the assay detects H_2O_2 accumulation during the period of incubation, marked differences could be seen over time between the control and the transgenic tissues (Figures 4B versus 4C and 4D versus 4E). The amounts of H_2O_2 in tissues of plants transformed with *GO* were two- to threefold higher than in the nontransgenic control plants, as determined biochemically by the titanium tetrachloride precipitation assays (Figure 5; Brennan and Frenkel, 1977). However, the transgenic plants grew normally under the growth chamber and greenhouse conditions and were phenotypically indistinguishable from the nontransgenic Russet Burbank potato plants.

Resistance of *GO*-Transformed Potato Tubers to Soft Rot Disease

Potato tubers contain glucose at levels ~1 to 2 μmol per gram fresh weight, and such levels increase substantially during storage in the cold (Sowokinos, 1990; Heineke et al., 1992). Thus, minitubers from transgenic and control lines were put into cold storage for one and a half months before use. Potato tubers were sliced into discs and tested for resistance to soft rot caused by *E. c. carotovora* under both aerobic and anaerobic conditions (Lapwood et al., 1984; Taylor et al., 1993). Nearly complete control of soft rot was observed in transgenic potato tubers of lines 22587-3 and 22587-34 (Figure 6A) when incubated aerobically with the bacteria. Tubers of untransformed Russet Burbank potato showed severe tissue maceration. Because *E. c. carotovora* causes the most severe tissue damage under near-anaerobic conditions (Pérombelon and Kelman, 1980), soft rot resistance of transgenic potato tubers was also evaluated anaerobically. Although the bacterial pathogen was more aggressive under such conditions, strong resistance to soft rot was sustained in tubers of transgenic potato lines (Figure 6B). Similar results were obtained when several other *GO*-expressing potato lines were tested for soft rot resistance (data not shown). In contrast, a vector-transformed control line, 17227-1, that had undergone the plant transformation process simultaneously with the *GO*-transformed lines, gave extensive tuber rot. Thus, resistance to soft rot in lines 22587-3 and 22587-34 was endowed by GO expressed from the transgene. Bacterial growth occurred initially on tubers of both transgenic and control lines. However, in transgenic tubers, the growth of bacteria was slower during the first 2 days of incubation and was strongly inhibited thereafter (Figure 7). As a result, there were nearly three orders of magnitude fewer bacteria per tuber disc in transgenic lines than in control lines by 3 days postinoculation.

Elevated Production of H_2O_2 Mediates Resistance to Soft Rot

As observed in leaf tissues, the amount of H_2O_2 in tuber tissues of transgenic lines 22587-3 and 22587-34 was determined to be two- to threefold higher than in the control lines. Interestingly, the amount of H_2O_2 in transgenic tubers increased an additional 1.5-fold 24 hr after the tubers were inoculated with *E. c. carotovora*, whereas little difference could be detected in tubers of control lines (Table 1). This result strongly suggests that elevated H_2O_2 levels mediate strong resistance to soft rot in transgenic tubers.

To determine whether H_2O_2 was required for bacterial resistance, we investigated the effect of catalase on disease progression in transgenic tubers. Catalase is a cellular AOS-scavenging enzyme that degrades H_2O_2 into water and molecular oxygen. At the time of inoculation, an excess amount of exogenous catalase was added to the potato tuber discs, and disease symptoms and bacterial growth were evaluated following 3 days of incubation under anaerobic conditions. As expected, the addition of catalase to tubers of the control potato lines had no significant effect on bacterial growth and tissue maceration (Table 2). In marked contrast, soft rot resistance of transgenic tubers was virtually abolished by the addition of catalase, leading to severe tissue maceration and substantial

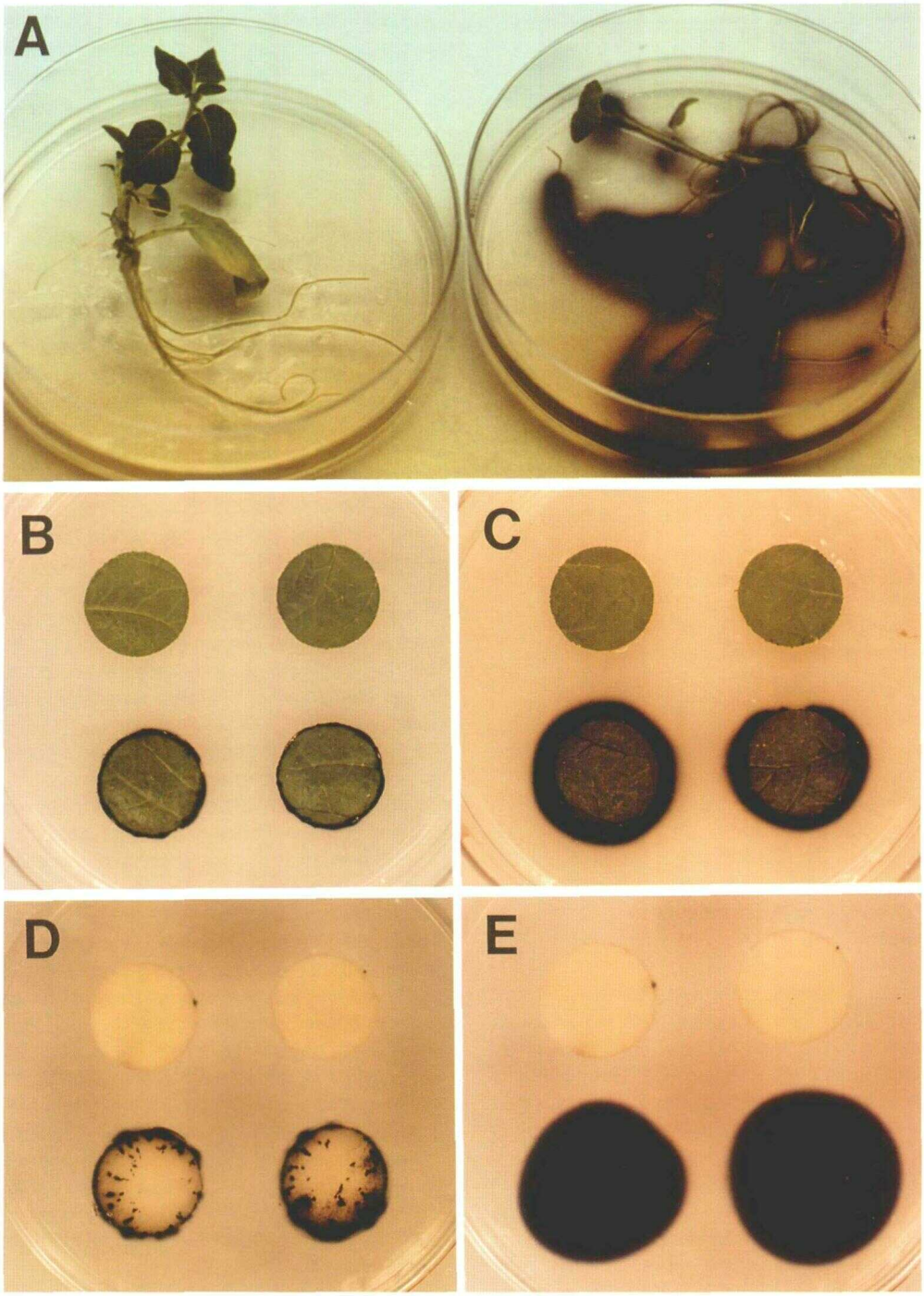

Figure 4. Detection of Elevated Production of H_2O_2 in Plant Tissues of *GO*-Transformed Potato on KI and Starch Medium.

(A) Roots of young potato plants placed in the medium for 2 hr. At left is nontransgenic Russet Burbank potato; at right, *GO*-expressing transgenic potato.
(B) and **(C)** Top view of leaf discs from the nontransgenic Russet Burbank (upper row) and GO-transformed potato plants (lower row). Leaf discs were allowed to developed for 30 min **(B)** and 5 hr **(C)**, respectively.
(D) and **(E)** Bottom view of tuber discs from the nontransgenic Russet Burbank (upper row) and *GO*-transformed potato plants (lower row). Tuber discs were developed for 30 min **(D)** and 5 hr **(E)**, respectively.

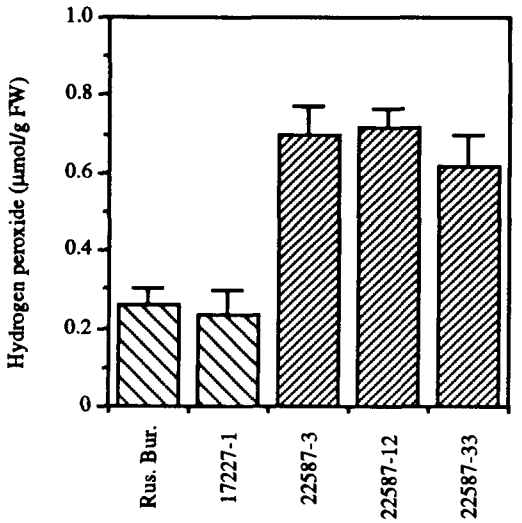

Figure 5. H_2O_2 Elevation in Leaf Tissue of Transgenic Potato Lines Expressing *GO*.

Levels of H_2O_2 were measured using leaf tissue of the nontransgenic Russet Burbank (Rus. Bur.) potato, vector-transformed control line 17227-1, and *GO*-transformed lines 22587-3, 22587-12, and 22587-33. The data represent the results of two independent experiments (n = 3). FW, fresh weight.

bacterial growth. These results suggest that soft rot resistance in transgenic tubers expressing *GO* is facilitated by H_2O_2, and its accumulation in tubers during infection is necessary for inhibition of the bacterial pathogen.

Resistance of Transgenic Potato Plants to Late Blight Disease

Because H_2O_2 also inhibits the growth of *P. infestans* in vitro (Figure 1), we assessed the resistance of *GO*-transformed plants to the late blight disease using both the detached leaflet assay and the whole-plant infection test. The symptoms caused by *P. infestans* on detached leaflets of *GO*-transformed lines progressed more slowly than on leaflets of the control lines (Figure 8). As a result, reduced rates of necrotic lesion growth were obtained from the infected leaflets of the transgenic lines (Figure 9). Within 6 days after inoculation, the average lesion sizes on leaflets of transgenic lines 22587-3 and 22587-12 were decreased by 56 and 49%, respectively, compared with that of the Russet Burbank nontransgenic control line. Comparable results were obtained when tests of late blight resistance were conducted using plants grown in the growth chambers. In addition to the significantly reduced percentage of diseased leaf areas, *GO*-transformed plants also showed very low numbers of dead petioles after infection with *P. infestans* (Table 3). Such enhanced resistance was not found in nontransgenic Russet Burbank potato plants. Several control lines that were transformed with the same plasmid vector but lacking the *GO* gene were also tested for late blight resistance. No significant protection against *P. infestans* infection was observed (Figure 9, Table 3, and data not shown).

DISCUSSION

We showed that expression of a gene encoding H_2O_2-generating GO confers resistance to bacterial as well as fungal pathogens in transgenic potato plants. The results demonstrate that AOS production can be manipulated in the plant through expression of a transgene and be linked to enhanced resistance of the plants to pathologically diverse pathogens. GO has previously been found to be the major antimicrobial metabolite of the fungus *Talaromyces flavus* used in the biological control of soil-borne pathogens (Kim et al., 1988, 1989), presumably through generation of H_2O_2. In potato, the effective control of both the bacterial soft rot disease and the fungal late blight disease occurred by virtue of elevated levels of H_2O_2. That H_2O_2 elevation accounts for the increased disease resistance shown by the *GO* transgenic plants is confirmed by our observation that, in the presence of catalase, resistance to soft rot could not be sustained (Table 2). Evidently, H_2O_2 generated by GO is an absolute requirement for rendering the transgenic plants disease resistant.

It was particularly striking that in transgenic potato tubers expressing *GO*, the levels of H_2O_2 increased even more after infection of the soft rot bacteria (Table 1). Biochemical genetic studies of the virulence of plant pathogenic *Erwinia* spp revealed previously that extracellular plant cell wall–degrading enzymes, such as pectate lyases and cellulases, play a central role in the pathogenesis of these bacteria (Collmer and Keen, 1986; Barras et al., 1994). Because most of the GO detected in transgenic plants was secreted into the intercellular space (Figure 2B), it is conceivable that action of these enzymes causes breakdown of the cell wall, thereby releasing intracellular glucose, which then serves as a substrate for the extracellularly expressed GO. Starch degradation, which yields glucose, also occurs during *Erwinia* infection of potato tubers (Stewart et al., 1994). Consequently, the level of H_2O_2 in tissue increases upon bacterial ingress and leads to growth inhibition of the bacteria. This notion is supported by the observation that the growth of the bacterial pathogen was inhibited after the initial increase during the first 2 days after inoculation (Figure 7).

The production of H_2O_2 and its related AOS during the pathogen- or elicitor-induced oxidative burst in plants has been hypothesized to be performed by a mechanism similar to the mammalian NADPH oxidase system (Vianello and Macri, 1991; Auh and Murphy, 1995). H_2O_2 elevation is extremely rapid and intense and is postulated to have multiple functions in the active defense of plant cells (Tzeng and DeVay, 1993; Mehdy, 1994; Baker and Orlandi, 1995) as well as in immune responses of animal cells (Schreck et al., 1991). The enhanced resistance

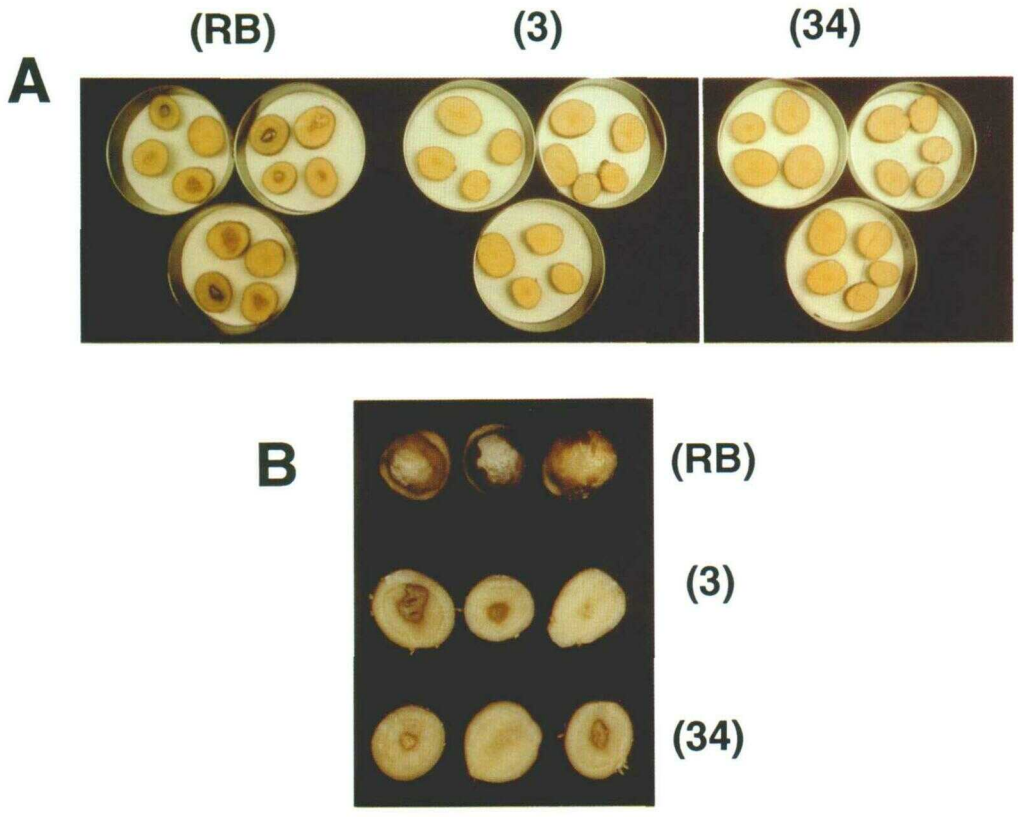

Figure 6. Soft Rot Disease Resistance Exhibited by Transgenic Potato Tubers Expressing *GO*.

(A) Inoculated potato tuber discs incubated at 23 to 24°C under aerobic conditions.
(B) Inoculated potato tuber discs incubated at 23 to 24°C under anaerobic conditions.
Potato minitubers of nontransgenic Russet Burbank (RB) and *GO*-transformed lines 22587-3 (3) and 22587-34 (34) were inoculated with *E. c. carotovora* at 2 × 10^5 cfu per tuber disc. Three days postinoculation, the disease symptoms were evaluated and photographs were taken.

to both a bacterial and a fungal pathogen exhibited by *GO*-transformed plants demonstrates the potential of obtaining broad-spectrum resistance by manipulating the endogenous levels of AOS in plants. This is supported further by our observation that transgenic potato plants expressing *GO* also exhibited strong resistance to a root fungal pathogen, *Verticillium dahliae*, that causes early die or wilt disease in potato (G. Wu, B. Shortt, and D. Shah, unpublished results). It is likely that increased levels of H_2O_2 in transgenic plants confer resistance to these diverse pathogens via a common mechanism.

The elucidation of the precise mechanism of H_2O_2-mediated resistance awaits further investigation. It is likely that the production of H_2O_2 has profound effects on both bacterial and fungal pathogenicity and plant defense. The antimicrobial activity of AOS, including H_2O_2, has been shown in vitro against plant pathogenic bacteria and fungi (Peng and Kuc, 1992; Kiraly et al., 1993). In this study, we also observed high sensitivities of *E. c. carotovora* and *P. infestans* to relatively low concentrations of H_2O_2 (Figures 1B and 1D). In addition to its toxicity to microbial pathogens, H_2O_2 is also thought to inhibit the activity of pectolytic enzymes, which are key pathogenic factors of *Erwinia* spp (Lyon, 1989). Moreover, evidence has shown that a certain threshold of bacterial growth on potato tubers is required before the virulence of *E. carotovora* can be fully expressed (Barras et al., 1994). The fact that H_2O_2 significantly suppresses bacterial growth on tubers of *GO*-transformed potato plants (Figure 7) suggests that the growth inhibition probably impedes the bacteria from exhibiting their pathogenicity.

H_2O_2 could also serve as a signal in turning on the natural defense mechanisms of potato plants during interactions with the pathogens. Recent studies using exogenously supplied O_2^- or H_2O_2 to leaf tissue or cells in suspension cultures have shown that these AOS trigger hypersensitive cell death (Adam et al., 1989; Levine et al., 1994), which often leads to systemic acquired resistance in plants. It was surprising that *GO*-transformed plants grew normally, although the levels of H_2O_2 in their tissues were elevated significantly (Figure 5 and Table 1). Apparently, these potato plants are capable of tolerating fairly high levels of H_2O_2 without developing visible growth abnormalities. However, it is also probable that the effects of H_2O_2 on plant tissue are different when it is generated by a

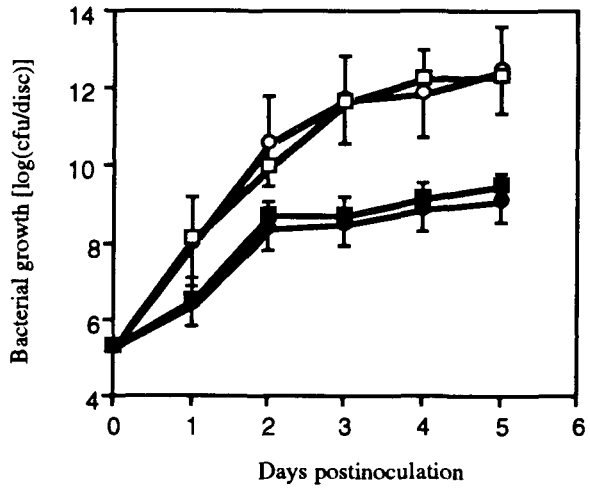

Figure 7. Growth Inhibition of *E. c. carotovora* on Tuber Discs of *GO*-Transformed Potato Lines.

Bacterial growth on potato tubers was measured using a group of tuber discs of each line at each day from 1 to 5 days postinoculation. Values obtained from potato lines of Russet Burbank (open circles), 17227-1 (open squares), 22587-3 (closed circles), and 22587-34 (closed squares) are indicated as means (n = 5) with standard deviations.

Table 1. H_2O_2 Elevation in Potato Tubers Expressing the *GO* Gene with or without Infection with *E. c. carotovora*

	H_2O_2 Level (μmol/g FW)[b]	
Potato Line[a]	Uninfected	Infected
Rus. Bur.	0.288 ± 0.060	0.254 ± 0.090
17227-1	0.278 ± 0.027	0.284 ± 0.016
22587-3	0.559 ± 0.050	0.861 ± 0.026
22587-34	0.745 ± 0.045	1.051 ± 0.052

Potato tuber discs were inoculated with sterile H_2O or a suspension of the bacteria and incubated under anaerobic conditions for 24 hr before being assayed for H_2O_2 using the titanium–hydroperoxide precipitation assay (see Methods).

[a] Potato lines are Rus. Bur., nontransgenic variety Russet Burbank; 17227-1, vector-transformed control line; and 22587-3 and 22587-34, two *GO*-expressing transgenic lines.

[b] H_2O_2 levels are indicated as micromoles per gram fresh weight (FW) of tuber tissues with sample standard deviations (n = 5).

constitutively produced enzyme localized in the apoplast, as compared with the rapid and intense burst of AOS that occurs during plant–pathogen recognition. Intracellular antioxidant mechanisms, such as peroxidases, catalases, and reduced glutathione, may counteract the effect of the extracellularly produced H_2O_2 in the transgenic plants.

H_2O_2 has been demonstrated to induce cross-linking of proline-rich proteins of the cell wall in soybean and broad bean (Bradley et al., 1992; Brisson et al., 1994). In addition, H_2O_2 has been found to influence gene expression in a number of defense-related cellular processes. H_2O_2 was found to stimulate phytoalexin production by potato tuber slices (Chai and Doke, 1987) and to induce glyceollin biosynthesis by soybean cells in suspension cultures (Apostol et al., 1989; Degousée et al., 1994). Increased accumulation of mRNAs encoding enzymes involved in phytoalexin biosynthesis, such as phenylalanine ammonia lyase, chalcone synthase, and chalcone isomerase, was observed (Mehdy, 1994). Moreover, the stability of certain defense-related mRNAs, such as the proline-rich protein mRNA in bean, may be regulated by AOS generated during host–pathogen recognition (Zhang et al., 1993). Finally, it has been shown that *PR-1* gene expression is induced in tobacco by elevated H_2O_2 levels after salicylic acid treatment or by injecting leaves with H_2O_2 (Chen et al., 1993). It will be interesting to determine whether these processes are affected in potato plants expressing *GO*.

The transgenic potato plants in which the level of H_2O_2 is elevated endogenously now provide us with a unique system to study more directly the involvement of H_2O_2 in plant defense processes. The use of an AOS-generating enzyme in plants also represents a novel and effective strategy of engineering disease resistance in agricultural crops. Given the nonspecific oxidative toxicity of H_2O_2 to a large number of plant microbial pathogens and its critical role in plant disease

Table 2. The Effect of Catalase on Soft Rot Resistance of Transgenic Potato Tubers Expressing *GO*

	Tissue Maceration (%)[b]		Bacterial Growth (cfu/disc)	
Potato Line[a]	+ Buffer	+ Catalase	+ Buffer	+ Catalase
Rus. Bur.	75.5 ± 14.2	88.1 ± 14.4	$1.85 (\pm 0.25) \times 10^{10}$	$1.85 (\pm 0.20) \times 10^{10}$
17227-1	78.2 ± 20.6	92.0 ± 14.8	$1.69 (\pm 0.21) \times 10^{10}$	$1.86 (\pm 0.14) \times 10^{10}$
22587-3	8.1 ± 3.0	85.6 ± 21.2	$1.67 (\pm 0.40) \times 10^{7}$	$1.59 (\pm 0.18) \times 10^{10}$
22587-34	5.3 ± 2.6	82.7 ± 24.2	$1.70 (\pm 0.90) \times 10^{7}$	$1.48 (\pm 0.18) \times 10^{10}$

Tuber discs were inoculated with *E. c. carotovora*, and Tris buffer or buffer containing catalase was added to each inoculation site. After 72 hr of incubation under anaerobic conditions, tissue maceration and bacterial growth were measured (n = 6).

[a] Potato lines are Rus. Bur., nontransgenic variety Russet Burbank; 17227-1, vector-transformed control line; and 22587-3 and 22587-34, two *GO*-expressing transgenic lines.

[b] Tissue maceration is indicated as a percentage of tissues per tuber disc rotted by the bacteria.

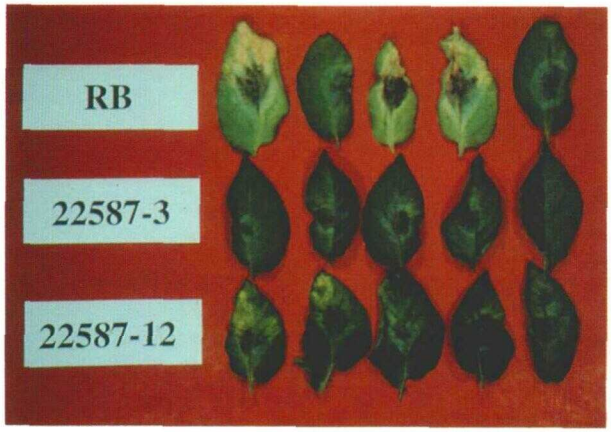

Figure 8. Resistance of *GO*-Transformed Potato Plants to Late Blight Disease Caused by *P. infestans* as Evaluated by Detached Leaflet Infections.

Leaflets detached from potato plants of nontransgenic Russet Burbank (RB) and two *GO*-transformed lines (22587-3 and 22587-12) were inoculated with *P. infestans* sporangia. The photograph was taken 4 days postinoculation.

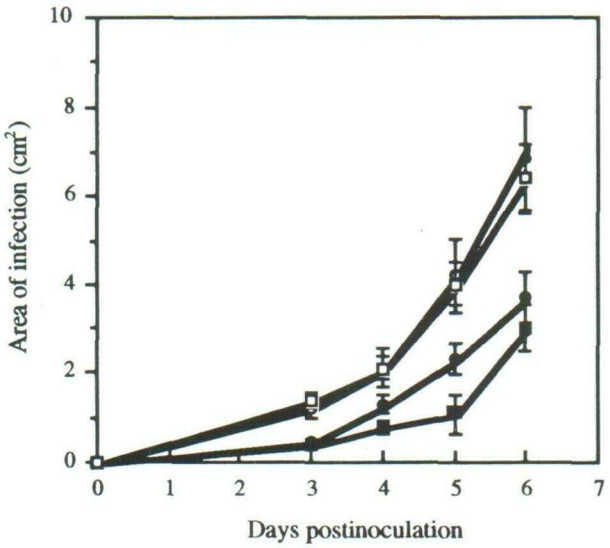

Figure 9. Reduced Rate of Lesion Growth on the Detached Leaflets of *GO*-Transformed Potato Lines Infected with *P. infestans*.

Detached leaflets were inoculated with sporangia of *P. infestans*. Areas of infected lesion size were measured each day after inoculation. Potato lines represented are nontransgenic Russet Burbank (open circles), vector-transformed control line 17227-1 (open squares), and two *GO*-transformed lines, 22587-3 (closed squares) and 22587-12 (closed circles).

resistance, the approach described here may yield broad-spectrum resistance to plant pathogens.

METHODS

Plasmid Construction and Plant Transformation

The gene encoding glucose oxidase (GO) was isolated from the genomic DNA of *Aspergillus niger* by polymerase chain reaction amplification using primers ANG-1 (5′-CCATCTAGAAGATCTATCATGCAGACTCTCCTT-3′) and ANG-2 (5′-TGGGGTACCGGATCCTTATCACTGCATGGAAGCATA-3′) designed according to the published sequence (Frederick et al., 1990). The *GO* gene was sequenced and cloned under the control of a figwort mosaic virus 35S promoter (Richins et al., 1987; Sanger et al., 1990) into the plant transformation vector pMON17227 containing a 5-enolpyruvylshikimate-3-phosphate synthase gene conferring glyphosate resistance (Barry et al., 1992), yielding the vector pMON22587. Potato (*Solanum tuberosum* cv Russet Burbank) was transformed via an *Agrobacterium tumefaciens*–mediated procedure (Newell et al., 1991) by selecting for glyphosate resistance. Several control transgenic lines were obtained by transforming potato with the vector pMON17227 lacking the *GO* gene. Independently transformed lines were designated by numbers after the plasmid construct name and propagated on tissue cultural medium (Newell et al., 1991) before planting in soil. Soil-grown plants were maintained in growth chambers with a 16-hr photoperiod of 60 to 70 $\mu E\ m^{-2}\ sec^{-1}$, at 21 and 16°C for day and night, respectively. Minitubers 2 to 4 cm in diameter harvested from the plants were stored in 100% relative humidity at 6°C for 1.5 months before use.

In Vitro Inhibition Assay

Cells of *Erwinia carotovora* subsp *carotovora* from overnight culture were washed with sterile water and diluted in potato dextrose medium to 10^5 colony-forming units (cfu) per mL before adding to a microplate. *Phytophthora infestans* race 0 was cultured on lima bean–V8 agar plates (Dhingra and Sinclair, 1985) at 19°C in the dark. Sporangia were collected from 2-week-old cultures in sterile water and diluted to 2×10^4

Table 3. *Resistance of Transgenic GO Plants to Late Blight Disease Caused by P. infestans*

Potato Line[a]	Leaf Disease (%)	No. of Dead Petioles
Rus. Bur.	12.3	2.2
17227-1	10.0	1.8
22587-3	3.5	0
22587-12	4.9	0.8
22587-33	6.3	0.8

Four leaves of potato plants were inoculated with sporangia of *P. infestans*. The percentage of diseased leaf area and the number of dead petioles of the infected plants were evaluated 7 and 14 days postinoculation, respectively. The LSD (0.10) of leaf disease is 7.1; the LSD (0.05) of number of dead petioles is 1.4.

[a] Potato lines are Rus. Bur., nontransgenic Russet Burbank potato; 17227-1, vector-transformed control line; and 22587-3, 22587-12, and 22587-33, three *GO*-expressing lines.

sporangia per mL. Sporangia were then germinated in a microplate for 48 hr. Various amounts of the *A. niger* GO or H_2O_2 were added to plates containing the bacterial cells or the pregerminated fungal sporangia, and the plates were incubated for 24 to 48 hr. The incubation temperatures for the bacterial and the fungal growth were 24 and 19°C, respectively. The growth of bacteria and fungal hyphae was monitored by measuring their absorbance at 595 nm using a microplate reader (Molecular Devices, Menlo Park, CA), and the percentage of inhibition by GO and H_2O_2 was calculated by comparing them with the water-treated controls.

GO Protein Detection and Enzyme Activity Analysis

Protein extraction from potato leaf or tuber tissues was performed in a buffer containing 125 mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol. Protein concentration in extracts was determined using the Micro BCA Protein Assay reagents (Pierce Chemical Co.). Intercellular wash fluid was collected by infiltrating leaves with 25 mM phosphate buffer, pH 7.0, containing 5 mM EDTA, and then by centrifuging at 500*g* for 10 min. Total protein extracts were subjected to SDS-PAGE, followed by electrotransfer to Immobilon-P membrane (Millipore, Bedford, MA). The immunoblots were developed with the antibody raised against *A. niger* GO using the enhanced chemiluminescence immunodetection procedure (Amersham). The amount of GO protein on immunoblots was quantified by comparing it with standards using the IS-1000 Digital Imaging System (Alpha Innotech Corp., San Leandro, CA). The GO enzyme activity assay was performed using the colorimetric procedure described by Gallo (1981). Briefly, protein was extracted from tissues by using 25 mM phosphate buffer, pH 7.0, containing 5 mM EDTA at 4°C. The protein extracts were incubated at 22°C in 70 mM of KH_2PO_4 buffer, pH 5.8, containing 0.57 mM of 4-aminoantipyrine, 0.35 mL/L of Triton X-100, 10 mM crystalline phenol, and 23 units per mL peroxidase and 175 mM glucose. The GO activity was measured by the absorbance at 510 nm using a microplate reader (Molecular Devices).

Detection of H_2O_2 in Plant Tissue

H_2O_2 produced by tissues of transgenic potato plants was detected essentially as described by Olson and Varner (1993). Roots of young potato plants or discs cut from leaves and tubers were placed in a medium containing 50 mM KI, 2 to 5% starch, and 1% agarose, and samples were left at room temperature to allow the color to develop. H_2O_2 level in plant tissue was measured using the titanium tetrachloride precipitation method as described by Brennan and Frenkel (1977), except that the absorbance at 405 nm of the titanium–hydroperoxide complex was read using the microplate reader. For leaf materials, tissues were extracted with 0.2 N $HClO_4$, and the extracted solution was passed through the AG-1 ion exchange column (Okuda et al., 1991) before being used for H_2O_2 quantification.

Potato Tuber Disc Assay for Soft Rot Resistance

Resistance of *GO*-transformed potato tubers was assayed using the tuber disc method (Lapwood et al., 1984; Taylor et al., 1993). Potato minitubers were sliced into discs ~1 cm thick, and each disc was inoculated with 10 μL of a suspension of *E. c. carotovora* containing 2 × 10^5 cfu in sterile water. In the experiment to determine H_2O_2 levels in tubers during bacterial infection, 300 μL of bacterial suspension containing 6 × 10^7 cfu was inoculated on each tuber disc to cover the whole surface. To determine the effect of catalase on soft rot disease progression, 10 μL of Tris-HCl, pH 8.0, buffer containing 20 μg of catalase was added to each inoculation site at the time of inoculation. The inoculated tuber discs were incubated at 23 to 24°C in Petri dishes containing water-saturated sterile filter paper at the bottom to provide moisture. For incubating inoculated tuber discs under anaerobic conditions, Petri dishes containing tuber discs were wrapped with Parafilm (American National Can, Greenwich, CT) and sealed in plastic bags. Three days postinoculation, tuber discs were weighed before and after removing the rotted tissue to evaluate the degree of tissue maceration. Bacterial titer on potato tuber discs was determined by suspending the macerated tissue in sterile water and plating a series of dilutions of the suspension on Luria-Bertani agar medium.

Potato Late Blight Resistance Test

Late blight resistance of *GO*-transformed potato plants was evaluated by both detached leaflet assays and whole plant infection tests. The detached leaf assays were performed as described by Liu et al. (1994) with slight modifications. Fully expanded leaflets (~20 cm^2) were inoculated by adding droplets of 100 μL of sporangium suspension of *P. infestans* to the center of the abaxial leaf surface. The inocula had a density of 10^5 sporangia per mL collected from 2- to 3-week-old plates containing lima bean–V8 medium (Dhingra and Sinclair, 1985). The inoculated leaflets were maintained in bioassay dishes (243 × 243 × 18 mm) (Nunc, Kamstrup, Denmark), with moisture provided by sterile water-saturated filter paper at the bottom, and incubated in growth chambers at 19°C with a 16-hr photoperiod. Symptom development was observed, and infected areas on the leaflets were measured by overlaying each leaflet with a 5 × 5 mm transparent grid. For each line of leaflets, the mean of infected areas and the standard deviations were calculated.

For the whole-plant infection test, transgenic and control plants were propagated by rooting stem nodes with a mature leaf and an axillary bud in a steam-pasteurized potting medium containing Metromix 200, sand, clay loam soil, and Redi-Earth (Grace-Sierra Horticultural Products Co., Milpitas, CA) in proportions of 5:2.5:1.25:1.25, respectively. Plants were grown at 19°C and 12 hr of light per day. An inoculum of *P. infestans* was prepared by adding sterile tap water to 10- to 14-day-old cultures grown in the dark on lima bean–V8 medium. When the test plants had grown 12 to 14 leaves >1 cm in width, they were inoculated by spraying the sporangial suspension onto the leaves until all were uniformly wet. Leaves were then placed in a mist tent for 48 hr at 19°C, with 12 hr of indirect light per day. Leaf wetness was maintained during the entire 48 hr. After an additional 5 days under normal (dry) growth conditions, disease severity was determined. Six plants of each test line were evaluated by estimating the percentage of diseased area on the center four leaves of each plant. At 14 days after inoculation, disease progress on the same four center leaves was reassessed by counting the number of leaf petioles that had been invaded and killed as the pathogen spread throughout the diseased leaves.

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