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Transgenic tobacco plants expressing the maize *Cat2* gene have altered catalase levels that affect plant-pathogen interactions and resistance to oxidative stress

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Abstract

Transgenic tobacco genotypes expressing the maize *Cat2* gene were developed with altered catalase (CAT) levels that resulted in a moderate increase of CAT activity in two transgenic lines. Bacterial infection, with a pathogen that does not share homology with the transgene, caused local and systemic down-regulation of the steady state mRNA levels of the 35S-driven transgene in a manner resembling post-transcriptional gene silencing (PTGS). Phenotypic symptoms of hypersensitive response (HR) and systemic acquired resistance (SAR) were similar in control SR1 and the transgenic genotypes. Induction of *hin1*, used as a molecular marker of plant responses to invading bacteria, displayed a similar pattern between control and transgenic lines, but some variation in the levels of expression was observed. The major difference was recorded in the ability of the plants to restrict bacterial growth during HR. All transgenic lines were more sensitive than control SR1, with two lines exhibiting a significantly reduced capacity to inhibit bacterial growth. This is consistent with the putative enhanced capacity of transgenic lines containing the maize *Cat2* gene to more effectively remove H_2O_2, which may act as a direct antimicrobial agent. Steady state mRNA levels of *PR-1* and *PR-5* varied among the genotypes, possibly indicating differences in strength of the SAR signal. Transgenic line 2, which was the most sensitive during HR, was most effective in restricting bacterial growth during SAR. This indicates that a reverse correlation might exist between the severity of infection during HR and the ability to inhibit bacterial growth during SAR. Growth under high light conditions affected plant-pathogen interactions in control SR1, as well as in transgenic line 8. Early induction and higher expression of *PR-1* and *PR-5* was detected in both SR1 and line 8 in high light-grown plants as compared with their low light-grown counterparts. Our data indicate that growth under high light conditions can predispose plants to better resist pathogen attack, and may amplify local and systemic defense signals. Finally, one transgenic line, which exhibited 1.3-fold higher average CAT activity in comparison with the untransformed SR1 control, suffered significantly less methyl viologen (MV) damage than untransformed control plants at moderate and high MV concentrations.

Abbreviations: PTGS–post-transcriptional gene silencing; HR–hypersensitive response; SAR–systemic acquired resistance; MV–methyl viologen; ROS–reactive oxygen species; SA–salicylic acid; SOD–superoxide dismutase; CAT–catalase protein/isozyme; *Cat*–catalase gene/transcript; HDGS–homology-dependent gene silencing.

Introduction

Aerobic organisms gain significant energetic advantage by using molecular oxygen as the terminal oxidant in respiration. However, they can be severely damaged by partially reduced oxygen species, which are produced through normal or aberrant metabolic processes, as well as a consequence of various environmental stresses. The toxic effects of reactive oxygen species (ROS), termed oxidative stress, are circumvented by a combination of enzymatic and non-enzymatic mechanisms that can reduce oxidative stress by con-

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verting ROS to harmless compounds. Among the enzymes involved in the defense against oxidative stress, catalase plays a key role by converting H_2O_2, at an extremely rapid rate, to oxygen and water (Scandalios, 1993).

Many plants subjected to a variety of environmental stresses respond by increasing the levels of mRNA and/or enzyme activity of antioxidant defense enzymes including catalase (Scandalios et al., 1997). Effective antioxidant defenses are crucial for survival, and understanding how they function is of great importance. Several recent studies have been aimed at enhancing protection by the constitutive overexpression of antioxidant defense enzymes in transgenic plants, including superoxide dismutases (SOD), which catalyze the dismutation of superoxide anion radicals to H_2O_2 and molecular oxygen, as well as glutathione reductase, which regenerates the antioxidant peptide glutathione, and ascorbate peroxidase, which scavenges H_2O_2 in chloroplasts and cytosol (Allen, 1995). Results from such studies confirm the important role antioxidant defense enzymes play in protecting plants from oxidative stress and point to the significance of transgenic plants as tools for the study of oxidative stress protective mechanisms *in vivo*.

The role of catalase has also been investigated using transgenic plants. Photosynthesis of transgenic plants expressing a bacterial catalase in the chloroplasts was found to be tolerant to high irradiance under drought conditions (Shikanai et al., 1998). Altered photosynthesis was also reported in transgenic tobacco plants expressing sense and antisense constructs of tobacco and cotton catalase (Brisson et al., 1998). Most studies focused on inhibition of specific catalase alleles using antisense constructs, or co-suppression mechanisms involved in transgene and endogenous homologous gene(s) inactivation by using sense constructs for transformation. Transgenic tobacco with reduced CAT activity developed necrotic lesions, and induced pathogenesis-related gene expression when grown under high light (Chamnongpol et al., 1996; Takahashi et al., 1997). Ion leakage from *Cat1*-deficient transgenic tobacco leaf disks was increased after 3 h of methyl viologen (MV; paraquat) treatment and was twice the levels of the control after 6 h (Willekens et al., 1997). A significant temperature-dependent increase of the CO_2 compensation point was observed in catalase-deficient transgenics, while the opposite was detected for overexpressing genotypes (Brisson et al., 1998). These studies point to the significance of catalase as a sink for photorespiratory H_2O_2, and its indispensable role for stress defense in C_3 plants.

Transgenic plants with altered catalase levels can be useful in examining the roles of catalase and H_2O_2 in HR and SAR. Microbial elicitors or attempted infection with avirulent strains of a pathogen cause the sudden rapid production of ROS (i.e., 'oxidative burst'), leading to the induction of HR, a rapid collapse of the challenged host cells, and the deployment of a battery of inducible defenses in both the challenged and the surrounding cells (Lamb et al., 1989). The result of hypersensitive cell death is the formation of necrotic lesions and the restriction of the pathogen to a small zone around the site of infection. An important consequence of HR is that the remaining uninfected tissues of the plant develop enhanced resistance against a second attack of the same, or even an unrelated pathogen, that can be long-lasting ('systemic acquired resistance'; SAR).

During the HR oxidative burst, a rapid accumulation of H_2O_2 at the plant cell surface is observed (Mehdy, 1994). In soybean cells, H_2O_2 accumulates within 2–3 min after delivery of the elicitor. The oxidative burst is transient, and begins to decline after 40–50 min. A dual role for the H_2O_2 burst in HR has been proposed (Levine et al., 1994): as a localized trigger of cell death, and as a diffusible signal for the induction of cellular protectant genes that function in blocking oxidant-mediated programmed cell death. The catalase inhibitor 3-amino-1, 2, 4 triazole enhanced cell death in soybean cells inoculated with an avirulent strain, but not in uninoculated cells, or in cells inoculated with the isogenic compatible strain of the pathogen *Pseudomonas syringae*. Transgenic tobacco plants constitutively expressing an antisense cDNA copy of the *Cat* gene exhibited reduced CAT levels and constitutively synthesized one class of pathogenesis-related proteins (Dempsey & Klessig, 1994). Thus, H_2O_2 has been proposed to serve as second messenger inducing plant defense gene expression.

A mechanism based on catalase inhibition by salicylic acid (SA) has been proposed as the cause for elevated H_2O_2 levels during HR and SAR, (Chen et al., 1993). However, it is unlikely that SA inhibition of catalase is responsible for all defense responses, since H_2O_2 accumulation is a rapid process, while SA accumulates within 8–24 h after inoculation (Dempsey & Klessig, 1994). SA involvement in the SAR response has been shown in transgenic tobacco expressing the bacterial salicylate hydroxylase (*nahG*) gene. These plants cannot accumulate SA; the

consequence is an inability to acquire SAR (Delaney et al., 1994). However, an untransformed top, grafted to a transformed rootstock expressing the *nahG* gene (unable to accumulate SA), did develop SAR when the rootstock was infected with TMV, even though it could not accumulate SA (Vernooij et al., 1994). Therefore, a mobile signal, other than SA, is required to act as second messenger for SAR development. Recent data show that H_2O_2-induction of SAR genes is dependent on SA accumulation (Neuenschwander et al., 1995) and that H_2O_2 does not function downstream of SA in the induction of *PR* protein expression (Bi et al., 1995). These data do not support a role for H_2O_2 in SAR signaling. The interplay of catalase and salycylic acid has also been investigated in crosses of transgenic plants that are catalase-deficient, with *nahG* transgenic plants that do not accumulate salicylic acid. In contrast with the parental catalase-deficient plants, the progeny do not constitutively express the *PR-1* gene or develop enhanced resistance, indicating that salicylic acid is required for the induction of defense responses in the catalase-deficient plants (Du & Klessig, 1997). However, SA-independent spontaneous lesion formation could be observed in several progeny genotypes under high light. These investigators conclude that there seems to be an SA-independent pathway for the formation of necrosis which is related to catalase deficiency and strong light, and an SA-dependent pathway leading to the induction of *PR* genes and enhanced resistance.

In order to further investigate the possible involvement and role of catalase in such important physiological phenomena as HR and SAR, as well as in protection against oxidative stress, we examined the effects of catalase over-expression in relation to HR and SAR. Accordingly, we developed transgenic tobacco genotypes, expressing the maize *Cat2* gene, with altered catalase levels. *Cat2* is not inhibited by SA, and is induced in SA-treated maize scutella (Guan & Scandalios, 1995). Herein we report results from experiments conducted to provide information on the effects of the additional catalase copies in transgenic genotypes in plant-pathogen interactions and in oxidative stress.

Materials and methods

Gene constructs

Cat2 cDNA was excised from the plasmid clone po*Cat2*.1c (GenBank accession J02976) as a 1570-bp *Eco*RI-*Sst*I fragment, containing part of the 5′ and the 3′ untranslated regions, and including the whole coding sequence of the *Cat2* cDNA. The 5′ *Eco*RI site was first filled in by Klenow polymerase, and the fragment then isolated by agarose gel electrophoresis.

The plasmid vector pBI.121 (Clontech) was reconstructed to transfer the *Cat2* cDNA into tobacco. pBI.121 derived from the binary vector pBIN19 (Bevan, 1984), contains an 800-bp CaMV 35S promoter fused to a 1.87-kb *gusA* gene with a 260-bp *Sst*I-*Eco*RI fragment containing the nopaline synthase (NOS) polyadenylation signal from the *Agrobacterium tumefaciens* Ti plasmid. The *gusA* gene was excised from pBI.121 as a *Sma*I-*Sst*I fragment, and the linear plasmid was isolated by agarose gel electrophoresis. The *Cat2* cDNA was then ligated into the plasmid vector in 5′-3′ orientation. The *Sma*I site of the vector was ligated to the *Eco*RI filled-in site of the *Cat2* cDNA and the *Sst*I site of the vector with the *Sst*I site of the *Cat2* cDNA. As a result, *Cat2* cDNA was properly inserted into the vector for the CaMV 35S promoter to drive its expression and NOS-terminator to add the polyadenylation signal. Orientation of the insert was confirmed by sequencing. The reconstructed vector was designated pBI.*Cat2*.

Tobacco leaf disk transformation

pBI.*Cat2* DNA was used to transform competent *E. coli* DH5αF' cells. The construct was then mobilized into *Agrobacterium* strain LBA4404 by the method of triparental mating (Bevan, 1984). Leaf disks of *Nicotiana tabacum* cv. SR1 were transformed as described (Horsch et al., 1985), except that a higher BAP/NAA ratio was used in the shoot regeneration medium. Transformed plants were selected on MS medium (Murashige & Skoog, 1962) containing 100 μg/ml kanamycin and 500 μg/ml carbenicillin. After rooting, plants were transferred to soil and grown in the greenhouse. Primary transgenics (R_0) were self-fertilized to produce R_1 seeds. R_1 seeds were germinated on kanamycin-containing agar plates and resistant plants grown to maturity in the greenhouse. R_1 plants were self-fertilized to produce R_2 seeds. Kanamycin resistant R_2 plants were selected on agar plates, transferred to soil, and grown under controlled conditions in the NCSU Phytotron. These plants served as the material for this study.

DNA isolation and Southern analysis

Genomic DNA from primary transformed tobacco plants and control, untransformed, SR1 plants was isolated (Dellaporta et al., 1983), digested with the appropriate enzymes, electrophoresed through 1% agarose gels and transferred onto nylon membranes. Presence of the transgene was detected by hybridization with a ^{32}P-labeled maize *Cat2* cDNA probe in Church buffer (Church & Gilbert, 1984), at 65°C overnight, and two washes for 30 min with 0.1X SSC/0.1% SDS at 65°C.

RNA isolation and analysis

Leaf material was harvested at the indicated stages, frozen in liquid nitrogen, and stored at − 80°C for analysis. Total RNA from leaf material was extracted (Thompson et al., 1983), and separated on denaturing 1.6% agarose gels. Equal loading was confirmed by ethidium bromide staining. RNA was then transferred to nylon membranes and hybridized with ^{32}P-labeled probes. Probes used were the maize *Cat2* full length cDNA, *hin1* (Gopalan et al., 1996), *PR-1* and *PR-5* (Ward et al., 1991), and *pHA2* (Jorgensen et al., 1987), containing an 18*S* ribosomal sequence as loading control. Hybridization was done in modified Church buffer (Church & Gilbert, 1984), containing 7% SDS, 0.5 M EDTA, 0.5 M NaH_2PO_4, and 1% BSA, at 65°C for 24–36 h. Filters were washed twice for 30 min with 0.1X SSC/0.1% SDS at 65°C. Duplicate northerns for each probe were prepared.

Catalase activity, protein determination, and western blot analysis

Leaf samples were homogenized in cold 25 mM glycylglycine buffer, pH 7.4. The crude supernatant was used for protein determination (Lowry et al., 1951) and catalase activity assay (Beers & Sizer, 1952). Catalase activity (reduction in absorbance at 240 nm/min/mg protein) was determined spectrophotometricaly. Western blotting was performed using maize CAT-2 specific polyclonal antibodies (Skadsen & Scandalios, 1987).

Plant material and growth conditions

The primary transformed lines (R_0) and the first transgenic generation (R_1) were grown to maturity in a greenhouse. R_2 plants were germinated and selected for 3–4 weeks on kanamycin plates, transferred to soil and grown in a controlled environment at the NCSU-Phytotron. The growing conditions were 12 h dark / 12 h light photoperiod at 25°C, 100 mol m^{-2} s^{-1} photosynthetic photon fluence rate, and 70% relative humidity. High light conditions were 500 mol m^{-2} s^{-1} photosynthetic photon fluence rate. Standard Phytotron nutrient solution was provided daily. Plants had 5–7 true leaves 30 days after transfer to soil. All treatments and analyses were at this stage, unless otherwise stated.

Pathogen infection and analysis

All experiments were conducted in two repetitions with five plants per genotype in each repetition. Bacteria used were *Pseudomonas syringae* pv. *syringae* isolate B728a and *P. syringae* pv. *tabaci* (ATCC 11528). Bacteria were grown in King's B medium (pH 7.0, 10 mg/ml protease peptone, 15 mg/ml glycerol, 1.5 mg/ml K_2HPO_4, and 4 mM $MgSO_4$). *P. syringae* pv. *syringae* was grown overnight in nutrient broth, centrifuged, and resuspended to an approximate concentration of 1×10^8 cfu/ml in distilled water. Leaves were infected with *P. syringae* pv. *syringae* by injection of leaf intracellular spaces using a 1 ml syringe without the needle. Each infiltration covered an area approximately 2 cm^2 and typically resulted in the application of approximately 100 µl bacterial suspension.

At 72 h after bacterial infiltration, the leaves were surface sterilized with 20% NaOCl, 0.1% Tween 20 for 1 min, and washed three times in sterile, distilled water. Leaf discs of 12 mm diameter were taken from the infection site, homogenized and plated. Two repetitions consisting of three plants per repetition (six leaf disks per genotype) were examined. Results were expressed as average number of colonies per leaf disk area at the appropriate dilution. Statistical significance of differences was examined by analysis of variance. No bacterial growth was observed on leaf discs obtained from mock-inoculated plants that were infiltrated with sterile water.

Seven days after initial treatment, SAR was induced by infecting the upper leaves of tobacco plants with *P. syringae* pv *tabaci* (ATCC 11528). Seventy-two hours after infiltration of leaves, bacterial growth was assessed as described above.

MV treatment and cell leakage analysis

MV belongs to the bipyridilium herbicide family. MV, as well as other amphiphilic viologens, can bind to

the thylakoid and mitochondrial membranes by hydrophobic interactions, and serve as an artificial electron carrier. During illumination, MV preferentially accepts electrons from photosystem I and donates them to molecular oxygen, generating the superoxide radical within the chloroplasts (Halliwell, 1984). Leaf disks (1.5 cm^2 each) were collected from transgenic and untransformed tobacco plants. Six leaf disks from each genotype were transferred to 35 mm Petri dishes containing 3 ml of MV solution of the following concentrations: 0.6, 1.2 or 2.4 μM. Control dishes contained distilled water. Each treatment was conducted in triplicate for each MV concentration and each genotype. Samples were vacuum infiltrated for 5 min and incubated at 21°C for 16 h in darkness. Leaf disks were then illuminated (500 μmol m^{-2} s^{-1}) for 2 h, and incubated in darkness at 30°C for an additional 16 h.

For cell leakage analysis, the solution on which the leaf disks had been floating was collected, made up to 3 ml (to correct for evaporation) and conductance was measured with a Markson model 1096 conductivity meter. The leaf disks were again floated on the MV solution, and incubated for 1 h at 65°C to release all solutes and the conductivity of the solution was measured again. The electrolyte leakage attributable to control and MV treatment was determined by dividing the conductivity value of the test sample by the conductivity of the sample after 1 h at 65°C.

Results

Introduction and expression of the maize Cat2 *gene in tobacco*

Transgenic tobacco plants expressing the maize *Cat2* gene were produced by introduction of a catalase cassette from the reconstructed vector pBI.*Cat2* (Figure 1A). Eight primary transformed kanamycin-resistant lines were analyzed for copy number of the transgene and found to contain 1–5 copies; one line (# 5) had no signal, indicating that the transferred *Cat2* gene was lost (Figure 1B). Expression of the maize *Cat2* in three transgenic lines was confirmed by slot-blot hybridization (Figure 1C). The RNA level produced in transgenic leaves under the control of 35S was comparable with that of maize leaves and epicotyls, but much lower than that of maize scutella at 4 d post-imbibition, when the highest level of *Cat2* transcript can be detected. No transcript was detected in the untransformed control, indicating that, under the hybridization conditions employed, no cross-hybridization of the maize *Cat2* cDNA with tobacco catalase transcripts occurred. Post-transcriptional processing of the maize *Cat2* transcript in tobacco leaves was confirmed by western blotting with the CAT-2-specific Ab. Immunodetection of CAT-2 protein in the transgenic lines indicated that six of the lines had similar CAT-2 content, which was slightly lower than that normally found in line W64A (Figure 1D). Line # 4 had significantly lower CAT-2 protein and line # 5, in which the introduced gene could not be detected, produced no CAT-2 protein. Line 5 was excluded from further experiments. No signal was detected in the untransformed control (SR1), confirming the specificity of the assay.

Variable levels of CAT activity were present in the transgenic lines depending on the generation (Table 1). CAT activity was determined from leaves of greenhouse-grown plants, and represents the mean of two experiments. The 5th leaf from the top of the plant, 30 days after transfer to the greenhouse (just before anthesis), was used for CAT activity. In the R_0 plants, lines 1 and 7 had higher CAT activity than that of the untransformed SR1, while the rest of the transgenics had lower CAT activity. The next transgenic generation (R_1) had significantly elevated CAT activity, and only one line (line 4) had lower activity than the control. This line had significantly lower CAT-2 protein in the primary transformants, as well. However, the generally higher CAT activity in the R_1 plants was not stable in the next generation (R_2), as only lines 7 and 8 had significantly higher CAT activity than the control SR1. Extended analysis of CAT activity in transgenic and wild type tobacco indicated that activity can vary widely depending on the age, type of tissue, light conditions, and nutrient supply (data not shown).

Plant-pathogen interaction relative to the expression of the maize Cat2 *in transgenic tobacco*

Physiological and molecular parameters of plant-pathogen interactions were examined in order to determine the role of the introduced *Cat* allele in the hypersensitive response (HR) and in systemic acquired resistance (SAR).

Hypersensitive response

Catalase transgenics and control SR1 plants were infected with *P. syringae* pv. *syringae* and progress of

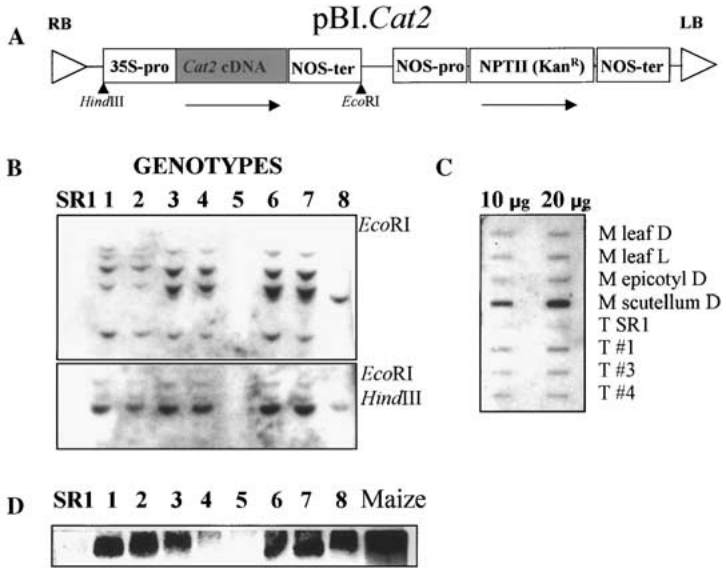

Figure 1. Transformation and expression of the maize *Cat2* cDNA in transgenic tobacco lines. **A**. The transformation vector pBI.*Cat2*. Arrows show the direction of transcription **B**. Southern hybridization with the maize *Cat2* cDNA probe of DNA isolated from control and transformed tobacco lines. Restriction only with *Eco*RI produced multiple bands in all but one line (#8), indicating incorporation of multiple copies of the transgene. Line #8 has a single copy. Double digestion with *Hind*III and *Eco*RI produced one band of the expected size in all lines. Line #5 did not produce a signal, indicating that it may have been a false positive and was excluded from further analysis. No signal is present in the control untransformed SR1. **C**. Slot-blot hybridization with the maize *Cat2* cDNA probe of total RNA from various maize (M) tissues grown under constant dark (D) or constant light (L) conditions, transgenic tobacco (T) lines, and untransformed control SR1. **D**. Immunodetection of the maize CAT-2 protein in the transgenic lines. No signal was observed in line #5, which did not hybridize with the *Cat2* cDNA in the Southern analysis, nor in the untransformed control line SR1. The maize band is included for comparisons.

Table 1. Leaf catalase (CAT) specific activity in transgenic tobacco lines transformed with the maize *Cat2* gene

Genotype	R_0		R_1		R_2	
	CAT activity	Samples	CAT activity	Plants	CAT activity	Plants
SR1	0.61 ± 0.24	4	0.58 ± 0.26	4	0.51 ± 0.23	11
1	0.94 ± 0.17	4	1.83 ± 0.31	4	0.58 ± 0.28	11
2	0.42 ± 0.16	4	1.38 ± 0.24	4	0.43 ± 0.13	11
3	0.18 ± 0.09	4	1.57 ± 0.12	4	0.46 ± 0.12	11
4	0.02 ± 0.00	4	0.42 ± 0.04	4	0.54 ± 0.21	11
6	0.32 ± 0.11	4	1.17 ± 0.19	4	0.54 ± 0.22	11
7	1.06 ± 0.32	4	1.51 ± 0.25	4	$0.71 \pm 0.23^*$	11
8	0.41 ± 0.15	4	1.36 ± 0.11	4	$0.68 \pm 0.32^*$	11

CAT specific activities ($\pm$ SD), expressed as units per milligram soluble protein, in three consecutive generations of seven families of transgenic tobacco plants and the untransformed control line, SR1. R_0, primary transformants; 4 samples per plant, grown in the greenhouse, were assayed just before anthesis, in duplicate experiments. In the selfed primary transformants (R_1) selected for kanamycin resistance, CAT activity was significantly higher, ranging from 2- to 3-fold that of SR1 in 6 lines; only line 4 was lower. Assays (with duplicate measurements) were performed in 4 greenhouse-grown plants (2 samples per plant) just before anthesis. In the next transgenic generation (R_2), derived from selfing of R_1 plants, CAT activity was determined in a wide range of developmental stages and conditions. Samples were taken from leaves of 45 days post-transplantation seedlings (2 plants @ 2 samples per plant), greenhouse-grown plants before anthesis (3 plants @ 2 samples per plant), and plants grown in controlled conditions (Phytotron) under low light ($100\,\mathrm{mol\,m^{-2}\,s^{-1}}$) (3 plants @ 2 samples per plant), or high light ($500\,\mathrm{mol\,m^{-2}\,s^{-1}}$) before anthesis (3 plants @ 2 samples per plant). CAT activity was determined in duplicate measurements as an average of 11 plants and 22 samples per genotype. Asterisks indicate statistically significant LSD from SR1 ($F = 1.98$, $p = 0.067$).

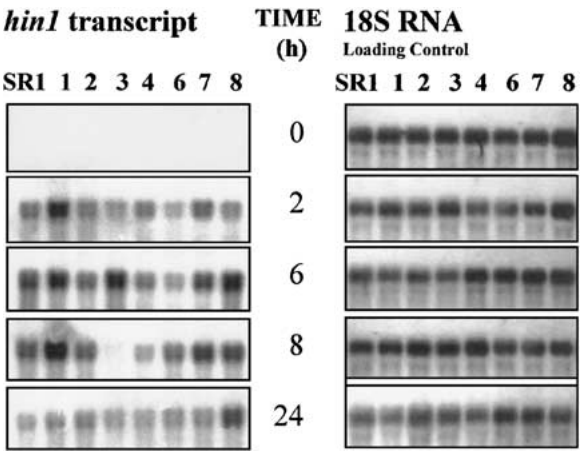

Figure 2. Expression of *hin1* in leaves of transgenic lines and in the untransformed control SR1 infected with the incompatible strain *P. syringae* pv. *syringae* at different time points during the progression of HR. Hybridization of the same filters with the *pHA2* probe containing an 18S ribosomal sequence was performed to ensure equal loading.

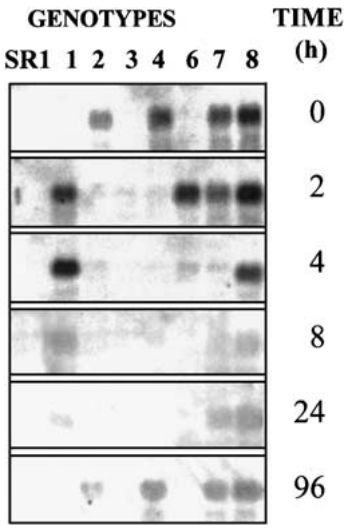

Figure 3. Expression of the introduced *Cat2* gene in transgenic tobacco lines during the progression of HR. Samples at time points 0 and 96 h are from uninfected leaves, while the rest of the samples are from infected leaves. No transcript is present in the untransformed SR1. The same set of filters was used also for hybridization with the *hin1* probe (time points 0–24 h) shown in Figure 2, and the *PR-1* probe (time point 96 h) shown in Figure 4 (*PR-1* d6). Equal loading was shown in Figure 2.

the HR was observed visually. Formation of necrotic lesions started ~8 h after infection; symptoms were indistinguishable between untransformed control and transgenic plants throughout the progression of HR. To determine if differences were present in the molecular events of HR, we monitored the induction of *hin1*, a plant gene activated rapidly by harpins and *avrPto* gene-mediated signals (Gopalan et al., 1996). The *hin1* transcript was not present in uninfected plants. Rapid induction of *hin1* was noted upon infection in wild type SR1, as well as in all the transgenic lines examined (Figure 2). The transcript was present at variable levels in all genotypes between 2–24 h after infection. The untransformed SR1 control displayed a typical *hin1* induction during the time course of the experiment, while the transformed genotypes displayed altered *hin1* expression, but with no unique pattern. Expression of the gene in line 1 was higher than the control SR1 at all time points and was the highest among all genotypes at 2 and 8 h post-infection. In line 2, expression was similar to the control SR1 at 2 h, slightly lower at 6 h, similar again at 8 h, and slightly higher at 24 h. In line 3 the *hin1* transcript was similar to the control SR1 at 2 h, higher at 6 h, significantly lower at 8 h, and similar at 24 h. Line 4 had similar transcript levels to the control SR1 at 2 h and 24 h, and lower at 6 h and 8 h. Line 6 had less transcript at 2 h, 6 h, 8 h, and slightly higher than the control at 24 h. Line 7 had a slightly higher transcript level at 2 h, similar at 6 h, and slightly higher than the control SR1 at 8 and 24 h. Line 8 had similar transcript levels to the control SR1 at 2 h, and higher thereafter.

In order to determine if expression of the introduced gene was affected, we monitored *Cat2* expression during the time course of HR using the same filter, after stripping off the *hin1* probe. We used the maize *Cat2* cDNA as probe, which specifically detected this transcript and detected nothing in the SR1 control (Figure 3). Expression of the gene was detected in uninfected plants in lines 2, 4, 7, and 8. In lines 1, 3, and 6 no transcript could be detected. Upon infection (2 h), the result was induction of the gene in lines 1 and 6, and in marked contrast, inhibition in line 4. At 4 h, the induction in line 1 was enhanced while in line 6 it was repressed. Expression in line 7 was also repressed, while in line 8 it declined slightly. Thus, 4 h after infection, only lines 1 and 8 were expressing the transgene. A significant reduction of transcript in lines 1 and 8 was evident at 8 h; this time point represents a stage in the progression of HR where the overall lowest level of expression of the introduced gene is detected. Very low transgene transcripts can be detected in genotypes 1, 7 and 8 at 24 h post-infection. We also examined the expression of the transgene in uninfected upper leaves 96 h post-infection. The expression pattern is similar to the one observed in uninfected plants, but at significantly lower level.

To determine if any relationship could be observed between this pattern of transgenic expression and bacterial survival in the infected tissue, we examined the number of bacterial-infected areas 72 h after infection. The lowest bacterial number was observed in the untransformed control SR1 (Table 2). Lines 1, 3, 6, and 8 had higher numbers of viable bacteria but the difference with the control was not statistically significant. Lines 2 and 7 had the highest numbers of viable bacteria, and differed significantly from the control SR1, but not from the other transgenic lines. These data indicate that killing of bacteria is more effective in the wild type SR1 plants than in the transgenic lines.

Table 2. Inhibition of bacterial growth during hypersensitive response (HR)

Genotype	Number of plates	Average colonies	Standard error
SR1	5	56.8	13.5
3	6	97.3	11.1
8	5	97.4	12.5
6	6	97.6	24.3
4	6	103.8	26.4
1	3	112.3	31.5
7	6	144.3*	10.8
2	5	168.6*	26.1

The average number of colonies of the incompatible strain *P. syringae* pv. *syringae* from leaf disk extracts of infected tissues 72 h after infection for each transgenic line and the untransformed control SR1. Asterisks indicate statistically significant differences from the control genotype SR1 ($F = 2.772$, $p = 0.0216$). Extracts were plated in a dilution of 10^{-6} on replicate plates.

Systemic acquired resistance

The ability of catalase transgenic plants to exhibit systemic acquired resistance was examined by monitoring the expression of *PR* genes in uninfected tissue at 4 days and 6 days after the primary infection with *P. syringae*. Results indicate that there are significant differences in the expression of *PR-1* and *PR-5* among all the genotypes examined (Figure 4). Expression of both *PR* genes is typical in the control SR1, with increasing transcript levels between day 4 and day 6 post-infection in systemic tissue. Transgenic lines display differences that are maximized at day 6, when lines 1 2, 4, and 7 have transcript levels lower than the control and lines 6 and 8 accumulate very high

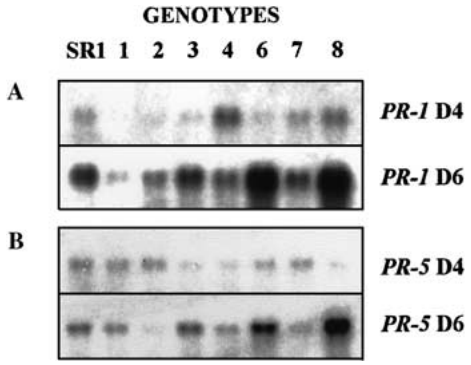

Figure 4. Expression of pathogenesis-related genes *PR-1* (**A**) and *PR-5* (**B**), four (D4) and six (D6) days post-infection with *P. syringae* pv. *syringae* used to induce HR, in transgenic lines and untransformed SR1. Samples were prepared from upper uninfected leaves. Equal loading was confirmed by hybridization of the same filters with the *pHA2* probe containing an 18S ribosomal sequence, with results similar to those displayed in Figure 2.

PR-1 and *PR-5* transcript, significantly higher than the control. Line 3 is at the level of the control.

The ability of the transgenic genotypes to express SAR as resistance to a second infection by a compatible pathogen was also estimated. Upper healthy leaves of plants previously infected with *P. syringae* pv. *syringae* were infected with the pathogenic strain of *P. syringae* pv. *tabaci* 7 days after the first infection. All genotypes were able to restrict the infection to the infiltrated area, exhibiting resistance to the pathogen. Symptoms were visually indistinguishable between control SR1 and transgenic plants. Bacterial survival was examined in the infected tissue 72 h post-infection (Table 3). The lowest bacterial count was observed in line 2 which had very high bacterial survival in HR. Lines 3, and 8 had the highest number of viable bacteria, and differed significantly from line 2. The other lines were intermediate and none differed significantly from the control.

Effect of light on pathogenesis-related gene expression in catalase transgenic plants

Catalase deficiency was shown to induce *PR-1* protein accumulation without pathogenic challenge in tobacco, but only after exposure to high light intensities (250–1000 $\mathrm{mol\,m^{-2}\,s^{-1}}$) which was accompanied by visible damage (Chamnongpol et al., 1996). To determine whether light has any effect on molecular events associated with SAR in our system, we examined *hin1* and *PR* gene expression in control SR1

Table 3. Inhibition of bacterial growth during systemic acquired resistance (SAR)

Genotype	Number of plates	Average colonies	Standard error
2	6	22.6	4.1
1	6	59.0	13.6
7	6	63.1	14.5
4	6	68.3	5.2
SR1	6	69.8	17.9
6	6	73.1	13.3
3	6	88.5*	21.1
8	6	99.3*	19.5

The second infection with the compatible strain *P. syringae* pv. *tabacci* was done 7 days after the primary infection with the incompatible *P. syringae* pv. *syringae*. The average number of colonies formed from leaf disk extract of infected tissue 72 h after the second infection for each transgenic line and the untransformed control SR1. Asterisks indicate statistically significant differences from genotype 2 ($F = 2.098$, $p = 0.0661$). Extracts were plated in a dilution of 10^{-5} on replicate plates.

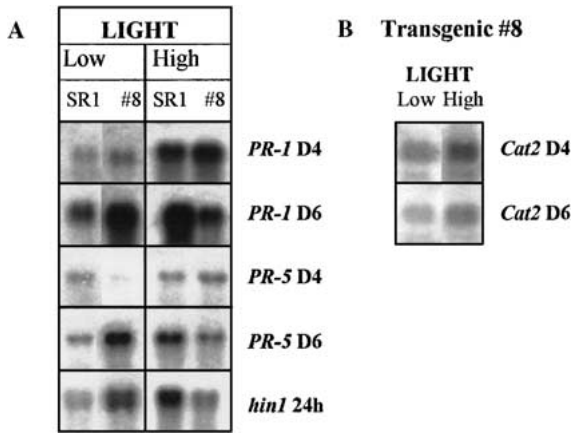

Figure 5. Effects of low and high light in expression of *PR-1*, *PR-5*, and *hin1* in SR1 and transgenic line #8, and *Cat2* in transgenic line #8 **A**. Effect of light on the expression of pathogenesis-related genes *PR-1* and *PR-5* four (D4) and six (D6) days post-infection with *P. syringae* pv. *syringae*, and the HR-specific *hin1* 24 h post-infection, in transgenic line #8 and untransformed SR1. **B**. Expression of the *Cat2* transgene in line #8 under low and high light conditions, four and six days post-infection. Equal loading was confirmed by hybridization of the same filters with the *pHA2* probe containing an 18S ribosomal sequence, with results similar to those displayed in Figure 2.

plants and transgenic line 8, which contains a single copy of the *Cat2* transgene, and showed the highest number of viable bacteria during SAR (Table 3). Plants grown under low ($100\,\mathrm{mol\,m^{-2}\,s^{-1}}$) or high ($500\,\mathrm{mol\,m^{-2}\,s^{-1}}$) light were infected with *P. syringae* pv. *syringae*, transferred to low light. The expression of *hin1* was examined in infected leaves 24 h later, while expression of *PR-1* and *PR-5* was examined in upper, uninfected leaves, 4 days and 6 days post-infection. In plants initially grown under low light, *PR-1* was detectable at low levels in both SR1 and line 8, at 4 days post-infection. At day 6 steady-state mRNA of *PR-1* increased in SR1, and greatly enhanced in line 8. The same pattern was observed for expression of *PR-5* in line 8. However, the difference in *PR-5* expression between day 4 and day 6 in SR1 was not significant (Figure 5A).

A different pattern was observed for plants grown under high light prior to infection. *PR-1* levels in both SR1 and line 8 were much higher than those observed under low light at day 4. Steady-state mRNA increased in SR1, while it slightly decreased in line 8 at day 6. This was in marked contrast with the *PR-1* expression observed under low light. At day 4, *PR-5* expression was no different in high light-grown plants than in their low light-grown counterparts. An increase in *PR-5* mRNA was detected under high light in SR1 at day 6, while there was no difference in line 8.

We also examined *hin1* expression in infected leaves 24 h post-infection. In plants grown under low light, *hin1* mRNA in SR1 was lower than that of line 8, while in plants grown under high light the reverse was true. In contrast, *hin1* mRNA in SR1 was much higher in plants grown under high light, while in line 8 it was higher in plants grown under low light.

In order to determine whether the patterns of *PR-1, PR-5,* and *hin1* expression in line 8, under low and high light, were related with any change in the *Cat2* transgene expression, we examined *Cat2* mRNA levels under the respective conditions. The *Cat2* transgene expression in line 8 was slightly higher under high than that under low light in the same upper uninfected leaves examined for *PR-1* and *PR-5* expression 4 and 6 days post-infection (Figure 5B).

The differences observed in molecular events in transgenic line 8 under low and high light were reflected in the ability of the plants to restrict bacterial growth after a second infection with the pathogenic *P. syringae* pv. *tabaci*. While this transgenic line harbored the highest number of viable bacteria under low light (average colonies 99.3, SE $\pm$ 19.5, Table 3), the number of surviving bacteria was significantly lower under high light (average colonies 49.5, SE $\pm$ 12.8).

MV resistance in catalase-overexpressing transgenic tobacco

Resistance of transgenic line 7 (exhibiting enhanced CAT activity in all experiments) to MV was determined by estimating electrolyte leakage as a measure of membrane damage. Leaf discs from the untransformed control SR1 showed increased sensitivity to the herbicide at 0–1.2 M, when membranes were almost completely disrupted. Leaf disks from the transgenic plants were significantly more resistant to the herbicide (Figure 6), showing 30% less damage than SR1 at 0.6 M, 45% less damage at 1.2 M and 25% less damage at 2.4 M. Similar experiments with the remaining transgenic lines revealed that increased resistance could be detected only at 1.2 M MV, and was of lower magnitude (20–30%) in comparison with 45% less damage in line 7 (data not shown).

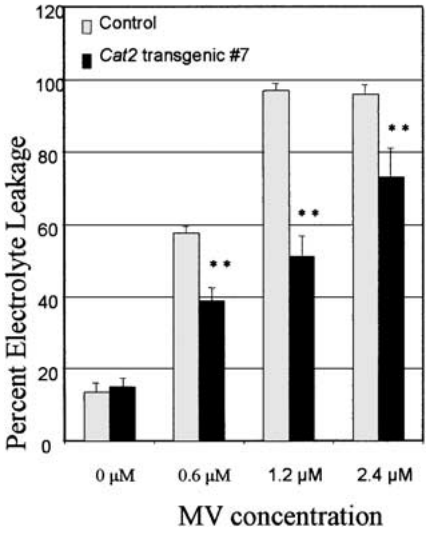

Figure 6. Percent electrolyte leakage (± SE) in leaf disks from the untransformed control SR1 and the transformed line #7 after treatment with the indicated concentration of MV. Six leaf disks of 1.5 cm^2 area from each genotype were assayed in triplicate experiments for each MV concentration. Samples were vacuum-infiltrated for 5 min and incubated at 21°C for 16 h in darkness. Leaf disks were then illuminated (500 $\mu mol\, m^{-2}\, s^{-1}$) for 2 h, and incubated in darkness for an additional 16 h at 30°C. The electrolyte leakage attributable to control and MV treatment was determined by dividing the conductivity value of the test sample by the conductivity of the sample after 1 h at 65°C. Double asterisks indicate statistically significant differences from the untransformed control ($F = 9.979$, $p = 0.0009$).

Discussion

Catalase expression in transgenic plants and their progeny

Catalase activity was enhanced in two primary transformed lines (1 and 7) and severely reduced in lines 3 and 4. In the remainder of the R_0 lines a moderate reduction in CAT activity was observed. Similar results have been reported in cotton (Chamnongpol et al., 1996), and tobacco (Brisson et al., 1998). These results might be explained by the action of a co-suppression mechanism (Matzke & Matzke, 1995). In R_1 six out of the seven transgenic lines exhibited CAT activity that was 2-3 fold higher than the untransformed control. Line 4, which had the most severe repression in R_0, was still lower in activity, but not as low as in the parental plants. In R_2 plants, CAT activity was comparable to that of the control SR1 and was higher in only two lines (7 and 8). This unstable inheritance of CAT activity in the transgenic lines strongly indicates the involvement of transgene inactivation, which is supported by the fact that most of the transgenic lines contained multiple copies of the transgene, while line 8 contained a single copy. It has been suggested that inactivation of gene expression in plants can result as a consequence of sequence duplications (Flavel, 1994). Northern analysis (Figure 3, time 0 h) revealed that there was no steady state *Cat2* mRNA in lines 1, 3, and 6, though it could be induced in lines 1 and 6, by pathogen attack. Thus, only line 3 showed no detectable steady state mRNA level of the *Cat2* transgene in our experiments, indicating a permanent inactivation of the transgene in this line. Lines 7 and 8 exhibited transgene expression and elevated CAT activity in the transgenic generation of our experiments (Table 1, R_2) and were considered as overexpressing lines. However, it must be noted that catalase activity and steady state mRNA levels are not always correlated. There are examples in maize (Redinbaugh et al., 1990) and cotton (Ni & Trelease, 1991) development where steady state mRNA levels do not reflect CAT activity or protein levels of different subunits, implying that post-transcriptional regulation is occuring. Our data are in accordance with previous results (Brisson et al., 1998) reporting a 1.3 to 1.5-fold increase in CAT activity over untransformed controls in transgenic tobacco transformed with a 35S-driven cotton and tobacco catalase cassettes, respectively. They also observed a lack of correlation between levels of RNA transcripts and

CAT specific activities in transformants overexpressing CAT.

Pathogen attack affects expression of the Cat2 *transgene*

In the course of our infection experiments we observed that in three transgenic R_2 lines (1, 3, and 6), a steady state *Cat2* mRNA could not be detected (Figure 3, time 0 h). However, while in line 3 transgene inactivation was permanent, in lines 1 and 6 there was an immediate induction of *Cat2* mRNA upon *P. syringae* infection, which declined during HR in infected leaves, and could not be observed 4 days post-infection in uninfected systemic leaves. The opposite pattern was observed in lines 2 and 4 where an immediate inactivation was detected upon infection, but a low level steady state *Cat2* mRNA was then present in systemic leaves 4 days post-infection. A similar but delayed inactivation was detected in lines 7 and 8, where very low steady state *Cat2* mRNA was observed 4 h post-infection in infected leaves, and 4 days post-infection in uninfected leaves. The above data derived from examination of four infected leaves in the same plants for the different time points, excluding random variation of transgene expression. Thus, we can identify at least four different transgene expression responses to invading bacteria among the seven transgenic genotypes examined:

(a) Immediate induction of steady state mRNA in silenced transgenes, followed by gradual silencing during the first 8 h post-infection (lines 1, 6);
(b) immediate silencing of previously active transgenes (lines 2, 4);
(c) gradual silencing of previously active transgenes during the first 8 h post-infection (lines 7, 8);
(d) no response of a silenced transgene (line 3). *Cat2* mRNA transcript could be detected 4 days post-infection in systemic uninfected leaves only in the genotypes that accumulated this transcript prior to infection, but at significantly reduced levels. These data indicate that transgene expression is affected by bacterial infection and its transcript is reduced 8–24 h post-infection.

Viral infection can cause stimulatory effects on a NOS promoter-regulated transgene and suppression on a 35S promoter-regulated transgene (Al-Kaff et al., 2000), suggesting that host responses to pathogen invasion may both up- and down-regulate transgenes. In transgenic plants where a transgene construct shares homology with the pathogen, the transgene can be silenced via a post-transcriptional gene silencing (PTGS) mechanism (Covey et al., 1997; Al-Kaff et al., 1998; Al-Kaff et al., 2000).

Although PTGS is a well-documented defense response to viral pathogens (Matzke & Matzke, 1995; Covey et al., 1997; Al-Kaff et al., 1998; Kooter et al., 1999) this is the first report providing evidence that bacterial infection (with a pathogen that does not share homologies with the transgene) causes local and systemic down-regulation of the steady state mRNA level of a 35S-driven transgene in a manner resembling PTGS.

Effects of altered catalase activity in plant-pathogen interactions

Catalase inactivation has been suggested as a primary step in SAR signaling during plant-pathogen interactions. The model suggests that CAT inactivation by SA enables the elevation of H_2O_2 levels and/or H_2O_2-derived ROS, which then serve as second messengers in the SAR signal transduction pathway (Chen et al., 1993). Thus, catalase should play a central role, serving as both receptor and transducer of the SA signal during plant-pathogen interactions. Consistent with this model are results from several studies indicating that H_2O_2 acts as a signal in *PR* gene induction (Conrath et al., 1995; Wu et al., 1995, 1997). However, this model has been questioned as results obtained by other investigators indicated that H_2O_2 acts upstream of SA in *PR* signaling (Bi et al., 1995; Leon et al., 1995; Neunschwander et al., 1995).

If catalase inactivation by SA is involved in HR induction, our transgenic plants expressing an SA-resistant CAT isoform should have shown altered HR responses. However, the phenotypic progression of HR was indistinguishable in untransformed control and transgenic lines. The induction of *hin1*, which is used as a molecular marker of plant responses to invading bacteria, displayed some variation between control and transgenic lines, but a similar pattern was observed. The major difference observed was in the ability of the plants to restrict bacterial growth. All transgenic lines were more sensitive than control SR1, with two lines (2 and 7) exhibiting a significantly reduced capacity to inhibit bacterial growth. This is consistent with the putative enhanced capacity of transgenic lines containing the maize *Cat2* gene to more effectively remove H_2O_2, which may act as a direct antimicrobial agent (Peng & Kuc, 1992), although

this finding is not consistent with the average CAT activity levels of these lines. Our findings are in agreement with the report that transgenic tobacco plants transformed with an antisense tobacco *Cat1* construct had significantly lower CAT activity, and were more effective in preventing growth of the incompatible *P. syringae* pv. *syringae* than the untransformed controls (Chamnongpol et al., 1996).

Regarding the effects of altered catalase expression in SAR signaling, we examined younger, uninfected tissue from inoculated plants for *PR-1* and *PR-5* expression. Detectable *PR* transcripts are produced in the younger uninfected leaves of tobacco between 3 and 6 days post-inoculation with TMV (Ward et al., 1991). *PR* activation was detected in control as well as in catalase transgenic plants in our study, indicating that the SAR signal transduction pathway was not disrupted by the expression of the *Cat2* transgene.

Steady state mRNA levels of *PR-1* and *PR-5* were variable among the different genotypes, possibly indicating differences in the strength of the SAR signal. It has been suggested that HR induces secondary oxidative bursts in discrete cells in distant tissues, leading to low-frequency systemic micro-HRs required for systemic immunity. In this way, H_2O_2 mediates a reiterative signaling network underlying systemic, as well as local, resistance responses (Alvarez et al., 1998). If the strength of the H_2O_2 signaling capacity is analogous to the H_2O_2 accumulation during the micro-HR oxidative burst, it is possible that even moderate changes in catalase activity might compromise the progression of SAR. This could explain the variable *PR* gene expression in the transgenic lines. Our data support a role of H_2O_2 in the signal cascade leading to SAR, as altered catalase gene expression, and consequent induction or suppression of CAT activity in the various transgenic lines, leads to variable *PR* gene expression.

Phenotypic expression of SAR involving the ability of plants inoculated with an incompatible pathogen to restrict a second infection with a compatible pathogen was also examined. Control SR1, as well as the seven transgenic lines, exhibited similar symptoms and ability to restrict the compatible pathogen *Pseudomonas syringae* pv. *tabaci*, at the inoculated area of upper leaves 7 days after the first infection with *Pseudomonas syringae* pv. *syringae.* However, bacterial growth estimated 72 h post-infection was significantly variable among the genotypes. The most effective in restricting bacterial growth was line 2, which was the most sensitive line during HR. This result indicates that a reverse correlation might exist between severity of infection during HR and bacterial growth inhibition during SAR. In support of this view is the fact that at the low end of bacterial growth were lines 2, 1, 7, and 4, which displayed lower steady state *PR*-gene transcript accumulation, and higher levels of bacterial growth during HR. At the high end were lines SR1, 6, 3, and 8, which displayed higher *PR*-gene mRNA levels, and lower levels of bacterial growth during HR. These data point to a possible relationship between severity of the primary infection and early induction of systemic responses.

In several studies light has been proven as a signal capable of inducing defense responses, as well as *PR-1* gene expression in CAT-deficient transgenic tobacco plants (Chamnongpol et al., 1996; Takahashi et al., 1997). We sought to examine the effects of light in our system, as well. Growth under high light conditions affected plant-pathogen interactions in control SR1, as well as in transgenic line 8 plants. At 24 h after inoculation with *Pseudomonas syringae* pv. *syringae*, the HR marker gene *hin1* was expressed at higher levels in control SR1, and lower levels in line 8, plants grown in high light, as compared to plants grown under low light. Early induction and higher expression of *PR-1* was detected in both SR1 and line 8 in high light-grown plants as compared with their low light-grown counterparts. Higher expression continued for SR1 at day 6, but for line 8 was lower than in the low light plants. The effects of light in *PR-5* expression were similar, but less pronounced, than those observed for *PR-1*. It should be noted that the effects concern plants grown first under high light and then transferred to low light for inoculation and further growth. Thus, our data indicate that growth under high light conditions can predispose plants to better resist pathogen attack, and may amplify the local and systemic defense signals. This is supported by the finding that the number of viable bacteria in transgenic line 8 plants grown under low light were significantly greater (average colonies 99.3, SE $\pm$ 19.5), than the number of surviving bacteria in plants grown under high light (average colonies 49.5, SE $\pm$ 12.8), during SAR inoculation with *P. syringae* pv. *tabaci*.

The inhibitory effect of pathogen inoculation on the 35S-driven *Cat2* transgene expression detected under low light can also be observed under high light. Steady state *Cat2* mRNA is lower at day 6 than that detected at day 4 under both light conditions. However, in plants grown under high light mRNA levels are slightly higher.

Resistance to oxidative stress

The ability of maize *Cat2*-expressing transgenic tobacco plants to resist oxidative stress was assessed in treatments with the redox-cycling herbicide MV, which intercepts electrons from various electron transport chains and transfers them to oxygen-generating superoxide. Most of this superoxide is subsequently converted to oxygen and H_2O_2 by the enzymatic action of superoxide dismutases. Attempts have been made to enhance MV tolerance by generating transgenic plants that overexpress different forms of SODs, and targeting them to various cellular compartments (Bowler et al., 1991; Sen Gupta et al., 1993; Slooten et al., 1995). However, these efforts have not always been successful (Tepperman & Dunsmuir, 1990). Also, efforts have been targeted at overexpression and elevation of levels of antioxidant enzymes responsible for reduction of H_2O_2. Again results have not been consistent, and have depend upon the gene used and the subcellular compartment to which the enzyme is targeted (Aono et al., 1991; Broadbent et al., 1995).

After examining the effects of MV on transgenic tobacco expressing the maize *Cat2* gene, our data are consistent with the hypothesis that this highly effective enzyme confers increased resistance to MV induced oxidative stress. Transgenic line 7 suffered significantly lower MV damage than untransformed control plants at moderate and high MV concentrations. This finding is consistent with results reporting enhanced MV-resistance in transgenic tobacco plants expressing the *E. coli katE* catalase gene, under control of the tomato *rbcS3C* promoter, targeted to the chloroplast (Shikanai et al., 1998). The rest of the transgenic lines were resistant only at low MV concentrations (data not shown). This was attributed to weak enhancement of catalase activity and/or transgene inactivation in several of these lines. Enhanced MV resistance of line 7 was detected with moderate elevation of mean CAT activity, supporting the significance of CAT as a cellular sink for H_2O_2 (Willekens et al., 1997). Catalase activity is likely regulated in multiple steps, one of which is the rate of transcription, but several post-transcriptional steps are also involved (Skadsen & Scandalios 1987; Scandalios et al., 1997). Increasing the rate of transcription is likely to speed up protein accumulation and to elevate CAT activity. Use of transgenes driven by the 35S promoter resulted in a small enhancement of CAT activity, as observed in this study and by others (Brisson et al., 1998). Perhaps the use of even stronger promoters will be necessary to attain high and stable CAT activity, in order to determine the magnitude of protection conferred by this enzyme under oxidative stress.

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