Antifungal and Peroxidative Activities of Nonheme Chloroperoxidase in Relation to Transgenic Plant Protection

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Nonheme chloroperoxidase (CPO-P) of *Pseudomonas pyrrocinia* catalyzes the oxidation of alkyl acids to peracids by hydrogen peroxide. Alkyl peracids possess potent antifungal activity as found with peracetate: 50% killing (LD_{50}) of *Aspergillus flavus* occurred at 25 μM compared to 3.0 mM for the hydrogen peroxide substrate. To evaluate whether CPO-P could protect plants from fungal infection, tobacco was transformed with a gene for CPO-P from *P. pyrrocinia* and assayed for antifungal activity. Leaf extracts from transformed plants inhibited growth of *A. flavus* by up to 100%, and levels of inhibition were quantitatively correlated to the amounts of CPO-P activity expressed in leaves. To clarify if the peroxidative activity of CPO-P could be the basis for the increased resistance, the antifungal activity of the purified enzyme was investigated. The LD_{50} of hydrogen peroxide combined with CPO-P occurred at 2.0 mM against *A. flavus*. Because this value was too small to account for the enhanced antifungal activity of transgenic plants, the kinetics of the enzyme reaction was examined and it was found that the concentration of hydrogen peroxide needed for enzyme saturation (K_m = 5.9 mM) was already lethal. Thus, the peroxidative activity of CPO-P is not the basis for antifungal activity or enhanced resistance in transgenic plants expressing the gene.

Keywords: *Aspergillus flavus; chloroperoxidase; fungal infection; peracid; plant disease; plant transformation; tobacco*

INTRODUCTION

Plants and animals generate hydrogen peroxide in response to microbial invasion (Doke et al., 1991; Jacks and Davidonis, 1996; Klebanoff, 1980). Hydrogen peroxide has antimicrobial activity, but much deadlier oxidants are formed from hydrogen peroxide in organisms that contain haloperoxidase (EC 1.11.1.10) (Klebanoff, 1980; Morrison and Schonbaum, 1976). For instance, animal myeloperoxidase and bacterial chloroperoxidase catalyze the oxidation of chloride by hydrogen peroxide to hypochlorite, which is up to 90-fold more lethal than is hydrogen peroxide to the fungus *Aspergillus flavus* (Jacks et al., 1991, 1999). Vascular plants lack haloperoxidase and consequently lack the corresponding system of antimicrobial defense. To determine the utility of nonplant haloperoxidase in plant antimicrobial defense, we transformed tobacco with a gene for chloroperoxidase (CPO-P) from *Pseudomonas pyrrocinia* and obtained greatly enhanced antifungal activity in tissue extracts in vitro and resistance to fungal infection in planta in primary transformants (Rajasekaran et al., 2000) as well as in their second- and third-generation progenies (unpublished observations). In the current study we substantiated the increased antifungal activity in transgenic plants but found that the peroxidative reaction catalyzed by CPO-P is not the basis for the effect.

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MATERIALS AND METHODS

Culture media were from Difco Laboratories (Detroit, MI). Hydrogen peroxide, obtained from Matheson Coleman & Bell (Norwood, OH), was quantified from its molar absorptivity of 67 M^{-1} cm^{-1} at 230 nm (Maehly and Chance, 1954). Peracetic acid and monochlorodimedon were obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals were obtained from commercial sources supplying the highest grades available. CPO-P enzyme was prepared from *P. pyrrocinia* as described previously (Wiesner et al., 1988), and the corresponding CPO-P gene was isolated from *P. pyrrocinia* as described earlier (Wolframm et al., 1993). Transformation of tobacco (*Nicotiana tabacum* cvs. Xanthi and SR-1) was accomplished using the *Agrobacterium tumefaciens*-mediated leaf disk transformation system (Horsch et al., 1985) as described previously (Rajasekaran et al., 2000). Plants regenerated from parallel transformation experiments with pBI121 but lacking the CPO-P gene served as controls in antifungal and enzymic analyses. Successful transformation was shown by PCR, Southern, northern, and western blot analyses (Rajasekaran et al., 2000).

Antifungal activities of leaf extracts of transformed and control tobacco plants were assessed in vitro following the method of De Lucca et al. (1997). Briefly, conidial suspensions of *A. flavus* were prepared from cultures grown on potato dextrose agar slants for 7 days at 30 °C. Conidial suspensions in 1% (w/v) potato dextrose broth (pH 6.0) were adjusted to 10^5 conidia/mL and germinated for 8 h at 30 °C prior to assays. Tobacco leaf extracts were prepared as described earlier (Rajasekaran et al., 2000). In brief, leaves were ground to a fine powder with liquid N_2, thawed homogenates were centrifuged at 8200*g* for 10 min at room temperature, and supernatants were tested for antifungal activity. Control samples were prepared from tobacco plants transformed only with pBI121. Conidial suspensions of *A. flavus* (25 μL) were added to 225 μL of supernatant, mixed, and incubated for 60 min at

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30 °C. Three 50 μL aliquots from each sample were then spread onto potato dextrose agar plates and incubated at 30 °C for 24−48 h and fungal colonies enumerated. Assays were conducted at least three times, and one-way ANOVA was used to determine the effects of extracts from transgenic plants on germinating conidia. Mean separations were performed using the method of Tukey (Sokal and Rohlf, 1981).

For tests of fungal lethalities with purified CPO-P and related metabolites in vitro, *A. flavus* was grown as described above. Conidia were harvested and incubated at 10^5 conidia/mL in 1% (w/v) potato dextrose broth at 30 °C to initiate germination. After 8 h, 50 μL of conidial suspension was added to 450 μL of 22.2 mM acetate buffer (pH 5.6) containing 0−1.1 M hydrogen peroxide. In experiments with CPO-P, 0.5 μg of CPO-P protein was added to each conidial suspension before conidia were added to the bioassay reaction mixtures. In some experiments 0.1 M NaBr or NaCl was included in bioassay mixtures. In other experiments bioassay mixtures contained 1 μM−10 mM peracetate as the oxidant. In this case, because peracetate preparations contained both hydrogen peroxide and acetate, their concentrations in bioassay mixtures were held constant at 4.7 and 18 mM, respectively, which were innocuous per se and corresponded to the amounts in the highest level of peracetate investigated. Bioassay reaction mixtures were incubated for 30 min at 30 °C. In some cases, incubation periods of up to 12 h were used and CPO-P was added to bioassay reaction mixtures rather than to conidial suspensions, but neither of these treatments affected results. Surviving conidia were assessed with corresponding statistical analyses by enumerating colony-forming units on potato dextrose agar as described earlier (De Lucca et al., 1995).

CPO-P activity was assayed by the decrease in absorbance at 290 nm occurring with the halogenation of monochlorodimedon by hypohalites (Morris and Hager, 1966). The enzymic reaction product of CPO-P, peracetate, spontaneously oxidizes bromide to hypobromite, which is quantitated with monochlorodimedon. Each enzymic reaction mixture contained 0.8 M acetate buffer (pH 5.6), 0.1 M NaBr, 10 mM NaN_3, 8 mM hydrogen peroxide, 50 mM monochlorodimedon, and enzyme source in a final volume of 3 mL (van Pée, 1996). When purified CPO-P was the enzyme source, 0.1−0.3 μg of enzyme protein was added. When leaf extracts were used, they were prepared with liquid N_2 as described above and diluted to 0.5 g of leaf tissue (fresh weight)/mL with 1.0 M acetate buffer. In experiments examining the effect of hydrogen peroxide on enzymic activity, hydrogen peroxide concentration varied from 0 to 60 mM. The rate of each enzymic reaction was linear, and proportionality was observed between each rate and the amount of enzyme when enzyme was rate limiting. A unit of enzymic activity is defined as the amount of enzyme that produces a decrease in absorbance at 290 nm of 0.001 per minute.

RESULTS

Tobacco plants were transformed with the CPO-P gene from *P. pyrrocinia* and tested for antifungal activity against *A. flavus*. Considerably more antifungal activity existed in leaf extracts of transformed plants than in extracts of control plants (Figure 1). When leaf extracts of control plants were spiked with CPO-P, the antifungal activity in the extracts increased to a level similar in magnitude to that in extracts of transgenic plants expressing the enzyme endogenously (Figure 1).

Leaf extracts of transgenic tobacco were tested simultaneously for inhibition of *A. flavus* growth in bioassays and for expressed CPO-P activity. Transgenic tissues contained both antifungal and enzymic activities, and the activities were quantitatively related. Figure 2 shows the correlation between the level of inhibition of fungal growth by leaf extracts and the amount of CPO-P activity expressed in the leaves.

The antimicrobial activity of the CPO-P reaction product, peracetate, and of the substrate, hydrogen peroxide, was assessed in vitro against *A. flavus*. According to the linear portion of the mortality curve in Figure 3, 50% killing (LD_{50}) occurred with ~25 μM peracetate. In marked contrast, the LD_{50} occurred at ~3.0 mM hydrogen peroxide and at ~2.0 mM when CPO-P was included in the bioassay (Figure 3). This indicates a 100-fold greater lethality of the enzymic reaction product of CPO-P compared to its substrate.

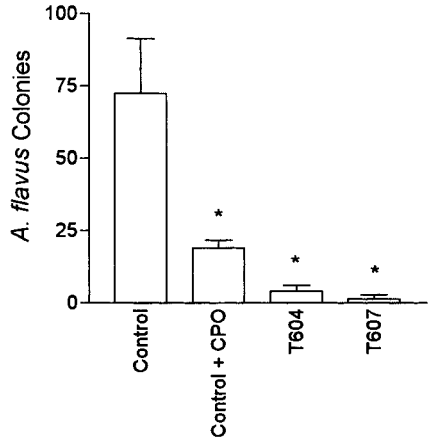

Figure 1. Inhibition of *A. flavus* growth by leaf extracts of transgenic tobacco (T604 and T607) and controls, one of which (control + CPO) was spiked with 0.5 μg of CPO-P protein. Asterisk (*) denotes significant reduction ($P < 0.05$) in the number of *A. flavus* colonies compared to unspiked control. Error bars indicate SEM ($n = 6$).

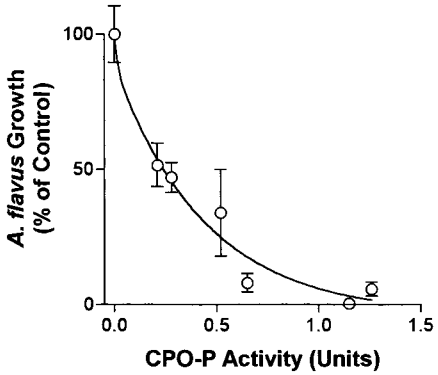

Figure 2. Inhibition of *A. flavus* growth by leaf extracts as a function of CPO-P activity in transgenic tobacco leaves. Vertical bars represent SEM ($n = 6$).

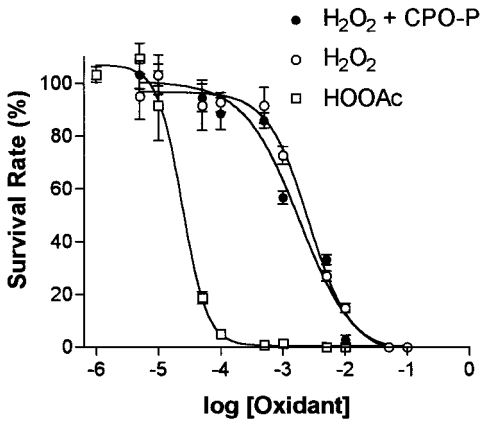

Figure 3. Effects of oxidants on the viability of *A. flavus* conidia. Oxidants are hydrogen peroxide, alone (H_2O_2) and in combination with CPO-P (H_2O_2 + CPO-P), and peracetate (HOOAc). Abscissa displays oxidant molarities. Vertical bars represent SEM ($n = 6$).

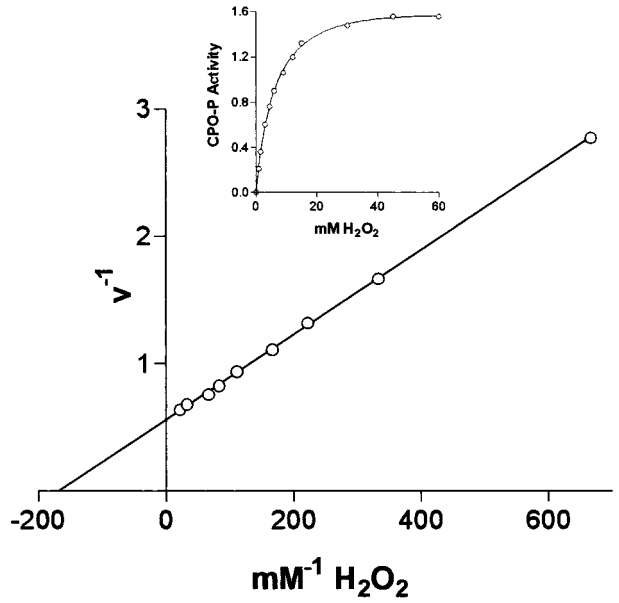

Figure 4. CPO-P activity as a function of hydrogen peroxide concentration (inset) and the corresponding Lineweaver−Burke plot. Data are the means of two independent measurements in duplicate, which do not deviate more than 5%.

The lack of an effect of CPO-P on the lethality of hydrogen peroxide (Figure 3) differed from the up to 90-fold increases in lethality produced by other nonplant haloperoxidases (Jacks et al., 1991, 1999). Inclusion of NaBr and NaCl in bioassay reaction mixtures had no effect on results, and CPO−P without hydrogen peroxide was not lethal (data not shown).

Because plants transformed with CPO-P exhibited enhanced antifungal activity in vitro and enhanced disease resistance in planta (Figures 1 and 2; Rajasekaran et al., 2000) yet the enzyme had virtually no effect on the lethality of its substrate hydrogen peroxide (Figure 3), we examined the kinetic properties of the enzymic reaction for elucidation. CPO-P activity as a function of hydrogen peroxide concentration is shown in the inset of Figure 4. A Lineweaver−Burke plot of the data (Figure 4) indicated an apparent K_m of 5.9 mM, clarifying the lack of effect of CPO-P.

DISCUSSION

Significant reductions in *A. flavus* viability by leaf extracts of tobacco transformed with CPO-P compared to leaf extracts from control tobacco (Figures 1 and 2) and inheritance of this trait among progenies suggest that CPO-P activity was the basis of the antifungal activity of the transgenic tissue. The quantitative correlation of the amount of antifungal activity in leaf extracts with the content of CPO-P activity expressed in the same leaves (Figure 2) supports this suggestion.

CPO-P catalyzes the oxidation of alkyl acids with hydrogen peroxide to form peracids (van Pée, 1996):

$$AcOH + H_2O_2 \rightarrow H_2O + AcOOH \qquad (1)$$

Ac is an acyl group. Ample hydrogen peroxide for the reaction is provided in plant tissues by disproportionation of stored superoxide (Jacks and Hinojosa, 1993) and by de novo synthesis upon infection (Jacks and Davidonis, 1996). The greater lethality of peracid to *A. flavus* compared to the lethality of hydrogen peroxide (Figure 3) furnishes additional support for the contention that the peroxidative activity of CPO-P is the basis for enhanced antifungal activity of transgenic plants compared to control plants.

However, the absence of an effect of CPO-P on the lethality of hydrogen peroxide (Figure 3) appeared to be incongruent not only with the large increases in hydrogen peroxide lethality effected by other nonplant haloperoxidases (Jacks et al., 1991, 1999) but also with the antifungal activity of leaf extracts from plants transformed with CPO-P (Figures 1 and 2; Rajasekaran et al., 2000). This absence of an effect by CPO-P is clarified from results of kinetic analysis of the enzymic reaction showing that the concentration of hydrogen peroxide needed for catalysis (K_m = 5.9 mM) (Figure 4) was already lethal to *A. flavus* (Figure 3). Consequently, the lethality of hydrogen peroxide could not be enhanced by CPO-P in bioassays.

CPO-P contains both peroxidase and hydrolase activities (van Pée, 1996). Our results show that the peroxidative reaction is not the basis of enhanced antifungal activity and resistance in plants transformed with CPO-P. Consequently, the hydrolase activity of the enzyme is more likely to be responsible. In this regard, it is of interest that CPO-P shows hydrolase activity with 3,4-dihydrocoumarin in the degradation pathway to salicylic acid (Kataoka et al., 2000), which is a signal for the mobilization of several defense systems (Delaney et al., 1994). In any case, CPO-P is directly involved as shown when extracts of inactive control tissues were spiked with purified enzyme and then acquired antifungal activity quantitatively similar to the strengths in extracts from transgenic tissues (Figure 1).

ABBREVIATIONS USED

Ac, acyl group; CPO-P, chloroperoxidase of *Pseudomonas pyrrocinia*; LD_{50}, concentration of a compound that kills 50% of fungi measured as reduction in formation of colonies; SEM, standard error of means.

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