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Evaluation of Peracid Formation as the Basis for Resistance to Infection in Plants Transformed with Haloperoxidase

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Nonheme haloperoxidase (HPO-P) isolated from *Pseudomonas pyrrocinia* catalyzed the peroxidation of alkyl acids to peracids. Among acids tested as substrates, acetic acid was most readily peroxidized. The reaction product peracetate possessed potent antifungal activity: 50% death (LD_{50}) of *Aspergillus flavus* occurred at 25 μM peracetate. Viability of *A. flavus* was inhibited by up to 80% by leaf extracts of tobacco plants transformed with the HPO-P gene from *P. pyrrocinia* compared to viability of fungi exposed to extracts from controls. To elucidate if peracid formation by HPO-P was the basis for antifungal activity in transgenic leaf tissues, lethalities of hydrogen peroxide−acetate−HPO-P combinations against *A. flavus* were examined in vitro. LD_{50} of *A. flavus* exposed to the combinations occurred at 30 mM acetate when concentrations of hydrogen peroxide and HPO-P were held constant. This value was identical to the LD_{50} produced by 30 mM acetate in the absence of hydrogen peroxide−HPO-P and therefore did not account for enhanced antifungal activity in transgenic plants. For clarification, kinetics of the enzymic reaction were examined. According to the concentration of acetate needed for enzyme saturation (K_m = 250 mM), acetate was lethal prior to its oxidation to peracetate. Results indicate that peracid generation by HPO-P was not the basis for enhanced antifungal activity in transgenic plants expressing the HPO-P gene.

KEYWORDS: ***Aspergillus flavus*; chloroperoxidase; haloperoxidase; peracetate; peracid; plant disease; plant transformation; *Pseudomonas pyrrocinia*; tobacco**

INTRODUCTION

Hydrogen peroxide is a microbicide generated by plants and animals in response to microbial invasion (*1*−*3*). It is also a reactant for the biosynthesis of deadlier microbicides in organisms that contain haloperoxidase (HPO) (EC 1.11.1.10) (*3*). For instance, animal and bacterial HPO catalyzes the peroxidation of chloride by hydrogen peroxide to produce hypochlorite that is 90-fold more lethal than is hydrogen peroxide to *Aspergillus flavus* (*4*, *5*). Vascular plants generate hydrogen peroxide but lack HPO and consequently lack the corresponding system of antimicrobial protection. To overcome this lack, we transformed plants with a gene for nonheme HPO from *Pseudomonas pyrrocinia* (HPO-P) (*6*, *7*). The resultant transgenic plants exhibited greater fungal resistance in planta and greater antifungal activity in vitro than the corresponding amounts in control plants. HPO-P catalyzes the generation of peracetic acid, a potent antifungal agent (*6*), from acetic acid and hydrogen peroxide (*8*). Whether formation of peracids such as peracetic acid by HPO-P is the basis for enhanced antifungal activity in transgenic plants was examined in this study.

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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 (*9*). 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. HPO-P was purified from *P. pyrrocinia* as described previously (*10*) and was the enzyme source used in all experiments. HPO-P was not purified from leaves. The corresponding HPO-P gene was isolated from *P. pyrrocinia* as described earlier (*11*). Transformation of tobacco (*Nicotiana tabacum* cvs. Xanthi and SR-1) was accomplished using the *Agrobacterium tumefaciens*-mediated leaf disk transformation system (*12*) as described previously (*7*). Plants regenerated from parallel transformation experiments with pBI121 but lacking the HPO-P gene served as controls. Results from PCR, Southern, northern, and western blot analyses and from enzyme assays showed that transformations were successful.

Antifungal activities of leaf extracts of transformed and control tobacco plants were assessed in vitro as described earlier (*13*). Briefly, conidial suspensions of *A. flavus* were prepared from cultures grown on potato dextrose−agar slants for 7 days at 30 °C. Conidial suspensions

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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 by Rajasekaran et al. (*7*). 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 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 (*14*).

For tests of fungal lethalities with purified HPO-P and related metabolites in vitro, *A. flavus* was grown as described above. Conidia were harvested and incubated at 10^5conidia/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 0−100 mM acetate buffer (pH 5.6). In experiments with purified HPO-P, 0.5 μg of enzyme protein and 0.1 mM hydrogen peroxide were added to each conidial suspension before conidia were added to the bioassay reaction mixtures. In some 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 not inhibitory and corresponded to the amounts in the highest level of peracetate investigated. Bioassay reaction mixtures were incubated for 30 min at 30 °C. Surviving conidia were assessed with corresponding statistical analyses by enumerating colony-forming units on potato dextrose−agar as described earlier (*15*).

HPO-P activity was assayed by the decrease in absorbance at 290 nm occurring with the halogenation of monochlorodimedon (*16*). Each enzymic reaction mixture contained 0.8 M acetate buffer (pH 5.6), 0.1 M NaBr or NaCl, 10 mM NaN_3, 8 mM hydrogen peroxide, 50 mM monochlorodimedon, and enzyme source in a final volume of 3 mL. When purified HPO-P was the enzyme source, 0.1−0.3 μg of enzyme protein was added. When enzyme activity was assayed in leaf extracts, 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 on the effect of substituting other buffers for acetate, buffer concentrations were 0.8 M (pH 5.6). In experiments on the effect of acetate on enzymic activity, acetate concentrations varied from 0 to 2.4 M and NaCl was added to maintain constant ionic strength at 2.1 M. The rate of each enzymic reaction was linear, and proportionality was observed between each rate and amount of enzyme when enzyme was rate limiting. A unit of enzymic activity is defined as the amount of enzyme that produces 1 μmol monobromomonochlorodimedon (MBMCD) min^{-1}.

RESULTS

Earlier we reported that transgenic tobacco plants expressing the gene for HPO-P from *P. pyrrocinia* exhibited enhanced resistance against *A. flavus* and other phytopathogens compared to resistance in control plants (*7*). For this study, we needed to assess antifungal activity in additional transgenic plants for verification. HPO-P activity was not detected in control plants but only in transgenic plants. As shown in **Figure 1**, survival of *A. flavus* was significantly reduced in conidia exposed to leaf extracts of transgenic plants expressing HPO-P (specimens T1−T7) compared to survival of conidia exposed to extracts of control plants lacking HPO-P (specimens C).

To shed light on how antifungal activity might be enhanced in transgenic plants expressing HPO-P, catalytic properties of purified HPO-P were examined. Brominating activity was observed with monochlorodimedon and NaBr to form MBMCD. Chlorinating activity did not occur under standard reaction conditions when NaCl was substituted for NaBr. Substitution of phosphate buffer for acetate buffer at identical pH values resulted in complete loss of activity, showing the requirement for an alkyl acid in the reaction. Several alkyl acids were tested as substrates for purified HPO-P as described under Materials and Methods. **Table 1** shows that the greatest affinity was for acetate. Because acetate was most readily peroxidized compared to the other alkyl acids, it was chosen as the substrate for further experimentation.

Lethalities of the HPO-P reaction product, peracetate, and its precursor, acetate, were assessed against *A. flavus* in bioassays. According to the linear portion of the mortality curve in **Figure 2**, 50% death (LD_{50}) occurred with ~25 μM peracetate. In marked contrast, LD_{50} occurred at ~30 mM acetate (**Figure 2**). These results indicate that a ~1000-fold greater lethality existed for the HPO-P reaction product, peracetate, compared to the acetate substrate. When HPO-P and hydrogen peroxide were added to acetate-containing reaction mixtures, LD_{50} still occurred at ~30 mM acetate (**Figure 2**).

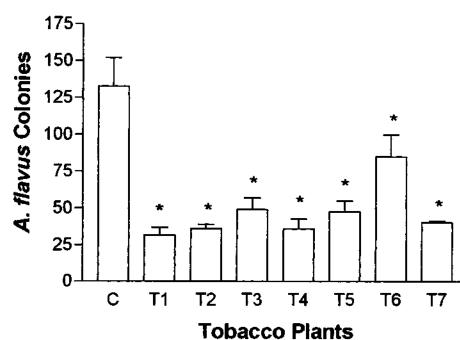


Figure 1. Inhibition of *A. flavus* growth by leaf extracts of control (C) and seven transgenic tobacco plants (T1–T7). Asterisk (*) denotes significant reduction ($P < 0.05$) in the number of *A. flavus* colonies compared to control. Error bars indicate SEM ($n = 6$).

Table 1. Substrate Specificity of HPO-P

substrate	specific activity[a]	comparative activity[b]
formate	0.01 ± 0.00	0.6
acetate	1.77 ± 0.02	100.0
propionate	0.94 ± 0.01	53.1
butyrate	0.35 ± 0.01	19.8
valerate	0.00 ± 0.00	0.0

[a] Micromoles of MBMCD formed/min/mg of protein. [b] Specific activity with acetate = 100.0

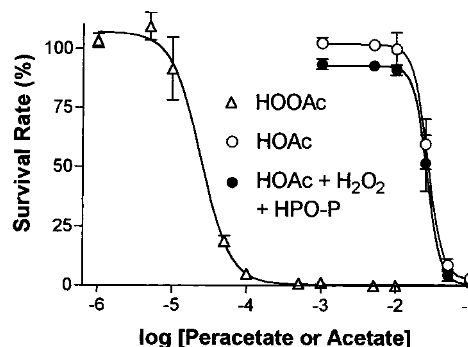


Figure 2. Effects of peracetate (HOOAc) and acetate concentrations on the viability of *A. flavus* conidia. Acetate was either alone (HOAc) or in combination with HPO-P and hydrogen peroxide (HOAc + H_2O_2 + HPO-P). Abscissa displays peracetate and acetate molarities. Vertical bars represent SEM ($n = 6$).

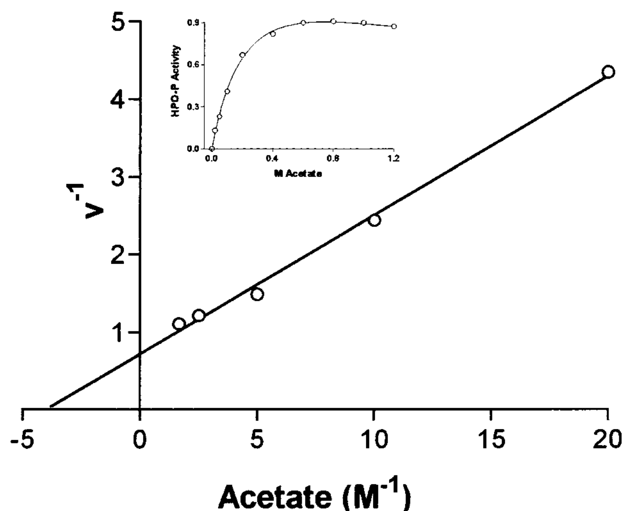


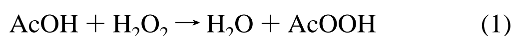
Figure 3. HPO-P activity as a function of acetate concentration (inset) and the corresponding Lineweaver–Burk plot. NaCl was included to maintain constant ionic strength at 2.1 M. Results are means of two separate determinations in duplicate in which values differed by <5%.

Because leaf extracts of plants transformed with the HPO-P gene exhibited both enzyme activity and enhanced antifungal activity (**Figure 1**), yet the enzyme had virtually no effect in vitro (**Figure 2**), we examined kinetic properties of the enzymic reaction for elucidation. Activity of purified HPO-P as a function of acetate concentration is shown in the inset of **Figure 3**. The Lineweaver−Burk plot of the data (**Figure 3**) indicated an apparent K_m of 250 mM, clarifying the lack of effect of the hydrogen peroxide−HPO-P mixture on acetate lethality.

DISCUSSION

Verification that transgenic plants expressing the gene for HPO-P gain resistance to microbial infection (*6*) was a goal of this work. HPO-P was chosen for transformation because the lack of heme and metal ion cofactors rendered post-translational processing for enzyme prosthetic groups unnecessary. Significant reductions of *A. flavus* viability by leaf extracts of transgenic plants compared to reductions by extracts of controls (**Figure 1**) verified enhanced antimicrobial activity and suggested that HPO-P activity was the basis for reduced viability.

Elucidation of the mechanism by which antifungal activity was enhanced by HPO-P was another goal of this study. HPO-P catalyzes peroxidation of alkyl acids to peracids (*8*)



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

where Ac is an acyl group. Acetate was the best alkyl acid substrate for the reaction among those that were tested (**Table 1**). The >10-fold greater value of the acidity constant, K_a, of formate compared to those for the other acids is consistent with a relatively small affinity of HPO-P for formate. Because the mechanism of the enzymic formation of peracid (eq 1) involves a hydrolytic-type reaction (*8*), the effect of chain length on the electronegativity of the acyl carbon explains results for acetyl and larger alkyls. The inverse relationship of alkyl chain mass to acyl carbon reactivity, the ponderal effect (*17*), is also consistent with these results.

The greater lethality of the enzymic reaction product peracetate to *A. flavus* compared to that of acetate (**Figure 2**) indicated that generation of peracids by HPO-P was the basis for enhanced antifungal activity in transgenic plants. The lack of an effect of purified HPO-P on acetate lethality (**Figure 2**), however, was incongruent with that proposition. This apparent discrepancy was clarified from results of kinetic analysis of the enzymic reaction. The concentration of acetate needed for catalysis (K_m = 250 mM) (**Figure 3**) was already lethal to *A. flavus* (LD_{50} = 30 mM) (**Figure 2**). Thus, even though peracetate appeared to be a potent fungicide (**Figure 2**) and transgenic plants expressing the HPO-P gene exhibited both enhanced fungal resistance in planta (*7*) and in vitro (**Figure 1**), our results indicate that peracid-generating activity of HPO-P is not the basis for fungal resistance in transgenic plants.

Because generation of alkyl peracids was not the cause of antifungal activity, identification of the basis is paramount. Besides possessing peroxidase activity, HPO-P also has hydrolase activity (*8*). This catalytic property might be responsible for fungal resistance. In this regard, it is of interest that an enzyme proteomically similar to HPO-P exhibits hydrolase activity with 3,4-dihydrocoumarin in the degradation pathway to salicylic acid (*18*). Salicylic acid is a signal for mobilization of antimicrobial defense systems in plants (*19*).

ABBREVIATIONS USED

Ac, acyl group; HPO, haloperoxidase; HPO-P, haloperoxidase of *Pseudomonas pyrrocinia*; LD_{50}, concentration of a compound that kills 50% of fungi according to decreases in formation of fungal colonies; SEM, standard error of means.

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