Soluble and Cell Wall Peroxidases in Reed Canarygrass in Relation to Disease Resistance and Localized Lignin Formation

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ABSTRACT

The relationship of peroxidases to an inducible disease-resistance mechanism involving lignification of leaf epidermal cell walls was studied. Reed canarygrass (Phalaris arundinacea L.) leaf discs were inoculated with Helminthosporium oryzae Eidam and floated on water. In inoculated discs, the activity of soluble, ionic wall-bound and covalent wall-bound peroxidases was about twice the level of activity in noninoculated discs. The increase was attributable to increases in activity of three cathodic isoperoxidases and to the appearance of a new cathodic isoperoxidase. Peroxidase activity in cryostat microtome sections of inoculated discs was histochemically localized in the wall near the site of attempted penetration. When inoculated discs were floated on solutions of cycloheximide (25 μg/ml), increases in peroxidase activity were inhibited, and the fungus penetrated the tissue. The inhibition of peroxidase activity was related to inhibition of cathodic isoperoxidase activity. Anodic isoperoxidase activity did not show changes in response to inoculation or cycloheximide treatment.

It was suggested that the resistance mechanism in P. arundinacea involves an induction of cathodic isoperoxidases in challenged tissue. These peroxidases may function in the biosynthesis of lignin at the site of attempted penetration.

MATERIALS AND METHODS

Reed canarygrass (Phalaris arundinacea L.) clone 6049 (9) was grown in a glasshouse bed of peatmoss-vermiculite. Leaf discs (8-mm diameter) were cut with a cork borer from unblanched, expanded leaves. The discs were floated on distilled H₂O or aqueous solutions of cycloheximide (25 μg/ml) in Petri dishes. The upper surface of the disc was thoroughly sprayed with a suspension of Helminthosporium oryzae Eidam spores or a 0.05-ml drop of spore suspension was pipetted directly onto the disc. The preparation of inoculum was described previously (16).

Cell-free extracts were obtained from samples collected 18 hr after inoculation. The samples were freeze dried. Cell wall and protoplast isoperoxidases were prepared as described by Birecka and Miller (1). Attempts to isolate free peroxidase from cell walls by vacuum infiltration were not successful. Protoplast and free wall peroxidases were isolated together as the soluble peroxidases. The assay for peroxidase was that described by Jennings et al. (6). Polyacrylamide disc gel electrophoresis as described by Davis (4) was used to separate anodic and cathodic isoperoxidases. The same amount of protein (400 μg) was applied to all tubes. Either 50 mM benzidine, 25 mM guaiacol, 25 mM o-dianisidine, or 25 mM pyrogallol was used as hydrogen donors to visualize isozyme bands. Gels were stained for 30 min, then 0.05% H₂O₂ was added to initiate the reaction.

Protein concentrations were determined by the method of Lowry et al. (7).

The histochemical localization of peroxidase was studied in 30-μm thick longitudinal sections of leaf discs cut with a cryostat microtome. Each section was assayed at 24 ± 2°C immediately after cutting. The section was placed on a microscope slide and covered with 2 drops of 50 mM pyrogallol solution (aq.) for 10 min. Then, 2 drops of 0.06% H₂O₂ were added. After 3 min, the solution was blotted away, and the section was rinsed with H₂O. The sections were observed at × 125 to 700 and photomicrographed on Ektachrome EHB 135 film immediately. Three controls were used: (a) pyrogallol omitted; (b) H₂O₂ omitted; and (c) inhibitor of catalase added. In the inhibitor controls, the pyrogallol solution was rinsed off with H₂O before 2 drops of inhibitor (1 mM NaCN or 1 mM NaNO₃) were added for 2 min. The inhibitor was rinsed off with H₂O, and H₂O₂ solution was added. Assays were run on two or more discs in each of three trials.

RESULTS AND DISCUSSION

Soluble and Cell Wall Peroxidases. Peroxidase activity in soluble fractions and ionic wall-bound fractions increased up to 2-fold in reed canarygrass tissue that was inoculated and incubated on water as compared to tissue that was not inoculated (Table I). The covalent wall-bound peroxidase showed an increase in relative activity in tissue inoculated and incubated on...
Table 1. Peroxidase Activity in Reed Canarygrass Leaf Disc Fractions after Various Treatments

Leaf discs were inoculated with a spore suspension of Helminthosporiumavenae as described earlier (16). The discs were floated on distilled water or cycloheximide solutions (25 μg/ml) for 18 hr before assay. All values are the mean of three experiments with three replications in each experiment.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Treatment</th>
<th>Peroxidase activities</th>
<th>ΔA/min/g mg protein/g</th>
<th>H2O2 binding</th>
<th>ΔA/min/g mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soluble</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Inoculated on water</td>
<td>221</td>
<td>65.25</td>
<td>3.38</td>
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<tr>
<td></td>
<td>Not inoculated on water</td>
<td>101</td>
<td>73.25</td>
<td>1.37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inoculated on cycloheximide</td>
<td>71</td>
<td>69.20</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>Ionic</td>
<td>Inoculated on water</td>
<td>77</td>
<td>24.60</td>
<td>3.13</td>
<td></td>
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<tr>
<td></td>
<td>Not inoculated on water</td>
<td>47</td>
<td>25.20</td>
<td>1.80</td>
<td></td>
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<tr>
<td></td>
<td>Inoculated on cycloheximide</td>
<td>24</td>
<td>25.50</td>
<td>0.94</td>
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<tr>
<td>Covalent</td>
<td>Inoculated on water</td>
<td>14.9</td>
<td>M</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Not inoculated on water</td>
<td>11.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inoculated on cycloheximide</td>
<td>9.5</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

1 Covalent protein could not be calculated because of cellulease and pectinase added to the preparation.

The data indicate that the cathodic isoperoxidases are the isozymes that are affected by attempted penetration. Inhibition of these isoperoxidases with cycloheximide is associated with the tissue becoming susceptible. Cathodic isoperoxidases of peroxidase in other plants show increases in response to injury or disease. Cathodic isozymes of corn (Zea mays) show increases in response to cutting injury and fungal infection (2). In Japanese radish (Raphanus sativus), a cathodic isozyme has been suggested as being the primary isozyme involved in regulation of lignin formation in response to disease (10). Our findings agree with these observations.

Histochemistry of Cell Wall Peroxidases. Histochemical tests of about 30 freeze-microtome sections from six discs taken from three experiments revealed a pyrogallol-oxidizing enzyme at the penetration site and in the adjacent outer epidermal wall within a radius of about 30 μm. The penetration site and associated wall area stained medium to bright amber when incubated with pyrogallol and H2O2 (Fig. 2A). The outer epidermal wall further away was not stained. In control sections incubated with pyrogallol alone (Fig. 2B) or H2O2 alone (Fig. 2C), the penetration area was light yellow as a result of a naturally occurring pigment (12). The adjacent epidermal wall was unstained. These results indicated that one or more peroxidases were active in the plant at the penetration site and in the nearby wall. The development of amber color at the penetration site and in the adjacent epidermal cell walls was inhibited by 1 mM NaCN, but was only slightly inhibited by 1 mM NaNO2. These data gave further evidence that a peroxidase was active at the penetration site (8). The tests also revealed peroxidase activity in guard cells and somewhat less activity in the prickle-hairs, veins, and mesophyll of normal and inoculated discs. Sections of leaves floated on cycloheximide solutions (25 μg/ml) and inoculated with H.avenae for 24 hr showed fungal penetration but did not show peroxidase activity at the point of penetration or in the epidermal wall around the site of penetration.

These data indicate that there was a localization of peroxidase activity in the cell wall area around the penetration site. Cycloheximide inhibited localization of peroxidase activity around the penetration site and allowed the fungus to penetrate the tissue. Earlier studies from our laboratory indicated that reed canarygrass produces a localized lignified papilla in the epidermal cell wall at the attempted penetration site (12) and fungi were not able to penetrate the epidermis (16). Treatment with cycloheximide inhibited the formation of these lignified papillae, and as a result, fungi not normally pathogenic to reed canarygrass could penetrate and ramify through the tissue (16). Cycloheximide

![Fig. 1. Relative activity of isoperoxidases from different fractions of leaf discs of reed canarygrass. The treatments are (a) discs incubated with Helminthosporiumavenae and incubated on H2O2, (b) discs noninoculated and incubated on H2O2, (c) discs inoculated with H.avenae and incubated on cycloheximide (25 μg/ml). Isozyme bands were stained with either 50 μM pyrogallol, 25 μM guaiacol, or 25 μM o-dianisidine. The reactions were initiated by adding 0.05% H2O2.](#)
that increased peroxidase activity in Japanese radish is involved in lignin formation which may be involved in resistance.

Data presented in this report are the first showing that inhibition of specific cell wall peroxidases around penetration sites results in susceptibility to nonpathogenic fungi. Studies are now underway to ascertain if inhibition of lignin and peroxidase occurs in a compatible host-pathogen association in reed canarygrass.

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LITERATURE CITED


Fig. 2. Cryostat microtome sections of reed canarygrass leaves inoculated with Helminthosporium avenae. The leaves show prominent apopositional growths (papillae = p) of the outer epidermal walls (w) surrounding the penetration channels (c) of the fungus. The fungal mycelium (m) was torn away during sectioning. A. Incubated with pyrogallol followed by H2O2. Deep amber color developed in the papilla and the surrounding 60-μm diameter area of the outer epidermal wall indicative of peroxidase activity localized in these areas. Walls farther away were not heavily stained. B. Control incubated with pyrogallol alone. C. Control incubated with H2O2 alone.