Transformation of potato with cucumber peroxidase: expression and disease response

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(Accepted for publication May 1988)

Peroxidase is thought to be important in a variety of plant defence responses against pathogens. Potato was transformed with a cucumber ascorbic peroxidase under the control of the cauliflower mosaic virus 35S promoter, which is highly expressed in potato tuber tissue. This particular peroxidase is associated with the induced resistance response in cucumber. High levels of transgenic peroxidase activity were expressed in tuber tissue. However, the increase in peroxidase activity had no effect on disease caused by Fusarium oxysporum, Erwinia carotovora, or Phytophthora infestans. Levels of soluble phenolics and polyphenol oxidase in the transgenic tubers were unaffected, while de amounts of lignin declined slightly.

INTRODUCTION

Lignification is a common response to plant infection. Lignin is a difficult polymer for pathogens to degrade and has been implicated in a variety of roles in plant defence [12]. In potato tuber (Solanum tuberosum L.) inoculated with the potato non-pathogen Cladosporium cucumerinum, lignin content increased about five-fold in 24 h, while pathogenic Fusarium oxysporum Fockel (teleomorph Gibberella pisi) (Fr: Fr) Sacc. caused only a two-fold increase [11]. This suggested that successful lignification was involved in stopping or slowing the course of infection. Detailed examination of Colletotrichum lagenarium in cucumber leaves showed that lignin develops as an infection barrier in cell walls and around hyphal tips, blocking their further growth [22]. Peroxidase is required for the final polymerization of phenolic derivatives into lignin. Activity of this enzyme increases greatly in potato tubers following infection with F. oxysporum [29]. Peroxidase is also implicated in suberization, a process that heals wounds with a layer of phenolic and aliphatic compounds [14]. Potato tissue produces several isomers of peroxidase [29] but the roles of most have not been identified.

When potato tubers are infected with F. oxysporum, the causal agent of dry rot, peroxidase activity increases dramatically in potato tubers [19]. Lignin levels in infected tuber tissue also increase substantially [19], and the margins of the lesions may

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0866-7254/98/000093-6 $30.00/0 © 1998 Academic Press
blacken, particularly if the lesion is contained. However, lignified zones may be breached and the infection then can develop further into the tuber.

It seemed probable that if some degree of lignification or the potential to lignify preceded inoculation, resistance would increase. To test this possibility, we introduced a peroxidase gene from cucumber (Cucumis sativus L.) [17] into potato, under the control of the cauliflower mosaic 3SS (CaMV) promoter which has been found to be highly expressed in potato tuber [8, 10]. This cucumber peroxidase is an extracellular acidic form that appears to be associated with resistance [27]. We regenerated transgenic potato plants and tested their tubers for peroxidase activity, lignification, and resistance to F. solani. We also tested leaf resistance to Phytophthora infestans (Mont.) de Bary, the cause of late blight, and tuber resistance to Erwinia carotovora subsp. carotovora (Jones) Bergey et al., the cause of soft rot.

MATERIALS AND METHODS

Plasmid construction

The cucumber peroxidase gene used was the largest of three acidic peroxidase genes cloned by Rasmussen et al. [27]. It was cloned in forward (sense) and reverse (antisense) directions into the BamHI site of Agrobacterium tumefaciens plasmid pBin19 [2] and transformed into A. tumefaciens. The plasmid with the sense gene was JR7, that with the antisense was JR6.

Plant transformation and regeneration

Leaves of greenhouse grown potato cultivars Snowden, Lembhi Russet, Superior and Desiree were transformed with A. tumefaciens following the procedure of De Block [4]. Shoots forming along cut edges were grown into plantlets which rooted freely on a medium containing kanamycin at 100 µg ml⁻¹. Some were tested by ELISA assay for the presence of neomycin phosphotransferase (NPTII) activity, following a protocol provided by 3 Prime-3 Prime Inc. (Boulder, CO). Rooted plantlets were transferred to the greenhouse and allowed to mature and form tubers. Leaves and tubers of primary transformants were used to assay peroxidase levels and disease responses.

Southern blotting

DNA was isolated from young leaves of tissue-cultured plantlets or greenhouse grown plants by a scaled-down version of the method of Dellaporta et al. [5]. Five to ten micrograms of DNA were digested overnight with 10 units of XbaI or EcoRI, then separated by electrophoresis on 0.7% agarose in Tris-acetate buffer. An alkaline blot procedure was used to transfer DNA to Zeta-Probe nylon membrane (Bio-Rad). Blotting and probing were carried out according to the manufacturer's protocol. The membranes were hybridized to the 32P-labelled JR7 plasmid which had been radiolabelled using a Biorad-Mannheim random priming kit.

Peroxidase and lignin assays

Peroxidase activity was assayed using leaf or tuber samples, which were extracted overnight in acetone at -20 °C. The acetone extracted samples were then dried, weighed, ground to powder, and suspended in phosphate buffered saline (pH 7.5). The
buffer extracts were frozen at -20 °C. After thawing, the debris was removed by centrifugation and peroxidase levels in the supernatant fluid examined. Total peroxidase activity was estimated using a guaiacol assay [3]. Nondenaturing anionic PAGE gels (7.5%, acrylamide, pH 9.3) were used to distinguish peroxidase isozymes and isoforms [27]. Gels were stained with 4-chloro-1-naphthol [59] and photographed. Intercellular fluid from leaves was extracted [58] and its peroxidase activity examined on gels.

Lignin content of tuber tissue was assayed by the thiglycollic acid method [11], except that tuber material was thinly sliced rather than ground. Samples for initial lignin levels were taken from freshly-sliced tubers, while samples from infected tubers were taken from 1-2 mm of tuber tissue adjacent to rotted zones.

Pathogen isolates

*Fusarium solani* strains used for testing dry rot resistance were R-6380 [7] and RN1, a thiabendazole-resistant strain [6]. They are about equally aggressive on potato tubers. *Phytophthora infestans* strain USA-8, isolated from Michigan fields in 1994, was used to test responses to late blight. *Erwinia carotovora* subsp. *carotovora* strain B15, was used to test responses to soft rot.

Inoculation and disease testing methods

To test responses to *F. solani*, newly harvested greenhouse grown tubers (2-8 cm in length) were washed, sliced into approximately 6 mm slices, and placed on damp filter paper in Petri dishes. The slices were immediately inoculated with 5 mm discs taken from the perimeter of growth of *F. solani* cultures grown on water agar. The lesions produced were examined and the width and depth of rot estimated by eye. In later experiments the widths were determined with calipers. Tissue prints onto nitrocellulose were stained for peroxidase activity [19]. To examine the suberization response of transgenic potato and its effect on *F. solani* infection, similar tubers of about equal size were sliced at intervals and placed on dry filter paper to suberize for different lengths of time. They were then inoculated with water agar discs from plates inoculated with *F. solani* RN1.

Toluidine blue O staining of fresh thin cross-sections of infected tubers was used to examine extent of lignification following wounding or infection [1].

Fully expanded greenhouse grown potato leaves of fairly uniform age were used to test resistance to *P. infestans*. Two cm leaf discs were placed adaxial side up on filter paper wetted with a 1 μg ml⁻¹ kinetin solution [23]. A 25-50 μl drop containing 2 × 10⁸ germinating sporangia of *P. infestans* was placed on each disc. Plates were incubated at 24 °C in a humid chamber. The extent of rot of each disc was scored as partial (0.5) or complete (1) and rot scores were recorded daily. Four to six leaves per plant were tested.

To test response to *E. carotovora*, strain B15 was grown overnight on Luria Broth agar and suspended in 50 mm KCl. Drops of 10 μl containing 4 × 10⁵ bacteria were placed on freshly-cut tuber surfaces. Tubers were incubated in a humid chamber at 24 °C for 2 days. Soft rotted tissue was wrapped away with a metal spatula and weighed.
RESULTS

Transgenic plants

Five to forty primary transformants of each cultivar/plasmid combination were obtained. About 30% of these were duplicates arising from the same transformation event, as indicated by Southern analysis. Nearly all material which rooted freely in the medium containing kanamycin tested positive in NPTII ELISA assay and Southern blots. Successful rooting in the kanamycin medium was therefore used to select putative transformants. Most of the adult primary transformants appeared normal in above-ground and tuber phenotype. A few plants of abnormal phenotype, such as abnormal leaf shape, were discarded.

Small samples of leaf tissue were examined on non-denaturing PAGE for the presence of additional bands of peroxidase. In a large number of plants transformed with JR7, one new band was visible. Additional forms with different glycosylation patterns may have been present. Since non-transformed potato leaf tissue has several isforms of peroxidase in the same size range, the new band could not be quantified or even distinguished in every case. No plants transformed with the JR6 plasmid (antiense peroxidase) showed any new bands of peroxidase activity.

After the potato plants had developed tubers, peroxidase was examined in the young tubers by non-denaturing PAGE and by measuring total peroxidase activity. Background levels of native peroxidase in these greenhouse-grown tubers were very low. The majority of potatoes transformed with the sense plasmid JR7 had very high levels of peroxidase, ranging from about 10 fold background levels to about 100 fold (Table 1). Some JR7 transformants, however (including some which were positive both on NPTII assays and on Southern blots) had levels within the background range (less than 2 fold average background levels). This included all the transformants of cv. Superior. A few had high peroxidase levels at harvest, but dropped to background levels after three months' storage at 4°C. Most, however, with high peroxidase activity at harvest, maintained high peroxidase levels after storage. No JR6 (antiense) transformants showed elevated levels of peroxidase. When isforms were observed on gels, tubers with high total peroxidase levels all showed three new bands of peroxidase against a background of almost no peroxidase (Fig. 1). These three bands presumably represent differently glycosylated forms of peroxidase and are the same sizes as in cucumber [17]. All plants which had additional leaf peroxidase bands had elevated tuber peroxidase and new tuber peroxidase bands.

The new peroxidase appeared to be exported to the extracellular space in leaf tissue, as all three forms of the transgenic peroxidase were enriched substantially in extracellular wash fluids (results not shown), as was observed in cucumber [21]. Extracellular fluids from tuber were not obtained. Peroxidase levels in cell wall fractions, however, did not increase significantly above background (results not shown).

Response to F. sambucinum

The very high peroxidase levels in some tubers suggested that significant differences might be found in their responses to tuber diseases. However, we found no evidence to suggest that increased peroxidase activity helps tubers resist F. sambucinum. At 4 days
Transformation of potato with cucumber peroxidase

### Table 1

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Number of transformants tested</th>
<th>Peroxidase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Average</td>
</tr>
<tr>
<td>Desire</td>
<td></td>
<td>2-2</td>
</tr>
<tr>
<td>JR6</td>
<td>6</td>
<td>2-5</td>
</tr>
<tr>
<td>JR7</td>
<td>5</td>
<td>0-5</td>
</tr>
<tr>
<td>Lemhi Russet</td>
<td>7</td>
<td>2-2</td>
</tr>
<tr>
<td>JR6</td>
<td>2</td>
<td>3-2</td>
</tr>
<tr>
<td>JR7</td>
<td>2</td>
<td>2-2</td>
</tr>
<tr>
<td>Superior</td>
<td>1</td>
<td>0-9</td>
</tr>
<tr>
<td>JR7</td>
<td>6</td>
<td>3-6</td>
</tr>
</tbody>
</table>

*SO$_{2}$, min$^{-1}$ (g dry weight)$^{-1}$

**Fig. 1**: Peroxidase in transgenic potato tubers. Peroxidase was isolated from tubers transformed with JR6 (antitoxin) or JR7 (vaccine strain). Inset sizes are the same as those produced in control; numbers in third row are identification numbers of individual transformants.

after inoculation with *F. arabidopsis*, the size of dry rot lesion on tuber discs did not correlate with peroxidase levels in any of the Snowdon, Lemhi Russet or Desire transformants (Fig. 2).

Similar results were observed when tubers were cut and allowed to suberize for different lengths of time prior to inoculation with *F. arabidopsis*. This cutting or suberization of a cut cortex surface for 2-4 days is essential enough to prevent *F. arabidopsis* establishment [9]. Desiree transformants with high peroxidase levels
developed suberization-related resistance to *F. solani* more quickly than did Desiree transformants with low peroxidase levels (both antiseptic transformants and sense transformants with no or low peroxidase expression) (Fig. 3). Lнемki Russet transformants, however, showed the opposite pattern; resistance developed slightly more slowly in tubers with high peroxidase activity. These data, drawn from relatively small numbers of tubers of non-uniform size and shape, were inadequate for statistical analysis but suggest that additional peroxidase expression did not increase tuber resistance.

Lignification was examined quantitatively by thioglycollic acid derivatisation [77].
Despite the high levels of peroxidase, total conservative amounts of lignin did not increase (Table 2). However, lignin content was slightly lower in tubers with high peroxidase activity than in those with low peroxidase activity. Toluidine blue O staining for lignified walls in infected tubers showed very similar lignification patterns for tubers with high or low peroxidase activity. Measurements of total lignin content in areas adjacent to lesions showed large increases in both low- and high-peroxidase tubers, but the increases were somewhat smaller in high-peroxidase tubers (Table 2). While this weak correlation between decreased lignin and increased peroxidase was consistent, considerable variability existed. This suggests that other restraints on lignification levels prevent additional lignification in uninfected tubers, or alternatively, that cucumber peroxidase is relatively inefficient at lignification compared with native forms, with which it may actually interfere.

The distribution of total peroxidase in transgenic tubers was examined by tissue printing [16] (results not shown). Peroxidase expression is high throughout the tubers, but particularly at or just under the periderm. Following wounding and infection, total peroxidase increased around the infection site, but not elsewhere. When recently-harvested tubers were infected, almost all peroxidase adjacent to lesions was the transgenic form (results not shown). If the transgenic peroxidase gene was present but not expressed, the induction pattern was the same as for nontransformed tubers of the cultivar. Previous studies of expression of the CaMV 35S promoter in potato showed high expression levels in a variety of potato tissues including tuber, together with little or no further induction on wounding or infection [18].

Because increased levels of soluble phenolic compounds were found in tomato transformed with a tobacco anionic peroxidase [18], we assayed total levels of soluble phenolic compounds in uninfected tubers and tubers infected with P. aphanis. We found a three-to-four-fold increase in soluble phenolic compounds on infection, independent of initial levels of peroxidase. Constitutive levels of polyphenol oxidase
TABLE 2
Lignin content of potato tuber tissue infected with P. sambucinum

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Plasmid/tuber</th>
<th>Uninfected†</th>
<th>Infected†</th>
<th>Range</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peroxidase activity</td>
<td>average</td>
<td></td>
<td>average</td>
<td></td>
</tr>
<tr>
<td>Lenzhi Russet</td>
<td>JR6</td>
<td>0.8</td>
<td>0.1-2</td>
<td>72</td>
<td>33-105</td>
</tr>
<tr>
<td></td>
<td>JR7 low activity</td>
<td>0.8</td>
<td>0.2-1.6</td>
<td>72</td>
<td>46-98</td>
</tr>
<tr>
<td></td>
<td>JR7 high activity</td>
<td>0.6</td>
<td>0.5-1.1</td>
<td>64</td>
<td>37-96</td>
</tr>
<tr>
<td>Desire</td>
<td>JR6</td>
<td>1.2</td>
<td>0.2-2.5</td>
<td>88</td>
<td>63-126</td>
</tr>
<tr>
<td></td>
<td>JR7 low activity</td>
<td>1.3</td>
<td>0.2-1.8</td>
<td>138</td>
<td>113-162</td>
</tr>
<tr>
<td></td>
<td>JR7 high activity</td>
<td>1.0</td>
<td>0.4-1.8</td>
<td>71</td>
<td>40-192</td>
</tr>
</tbody>
</table>

*Peroxidase activities were classed as low if less than 3 x the background level, high if above this level.
†Lignin content determined by the thioglycolic acid procedure and is expressed as mg mg\(^{-1}\) dry weight. Samples of infected tissue were taken at 4 days after inoculation from areas adjacent to rotted tuber tissue.

![Graph showing response of transgenic potato leaves to P. infestans infection. Degree of resistance determined as explained in Materials and Methods, at 3, 4, and 5 days after inoculation. Desirée without peroxidase, □; Desirée with peroxidase, ■; Lenzhi Russet without peroxidase, ○; Lenzhi Russet with peroxidase, ▲. There is no apparent correlation between peroxidase concentration and response to P. infestans.](image)

and of soluble phenolics were also independent of initial peroxidase levels (results not shown).

Response to P. Infestans
Leaf discs of transgenic potato lines were challenged with P. infestans. The discs quickly developed spreading lesions, which destroyed the leaf discs within 4–5 days after inoculation. Leaf discs of Desiree rotted slightly more quickly than those of Lenzhi Russet (Fig. 4), but the presence of high peroxidase levels did not affect the rate of...
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![Graph showing peroxidase activity](image)

Fig. 5. Weight of soft rot produced on transgenic tubers inoculated with *E. carotovora*, at 2 days. Peroxidase is measured in MOE4 min−1 (g dry weight)−1. There is no apparent correlation between peroxidase concentration and response to *E. carotovora*.

decay significantly in either cultivar (Fig. 4). Although tuber peroxidase levels might not correlate tightly with leaf peroxidase levels, we found that leaf peroxidase bands on gels always correlated with tuber peroxidase bands, and their absence in leaf material with their absence or near-absence in tuber material. The bulk of these results suggest that additional peroxidase expression does not help potatoes fend off *P. infestans*.

**Response to *E. carotovora***

Tuber slices infected with *E. carotovora* developed deep soft rot within two days. The weight of rotted tissue was no different in Lemhi Russet or Desirée transformants with either JR6 or JR7 (Fig. 5). Elevated peroxidase levels are therefore of little utility against this bacterial species.

**DISCUSSION**

Our initial expectation was that increased peroxidase levels in potato tubers would increase their potential to synthesize lignin and consequently enhance resistance to *Fusarium oxysporum*, and possibly to other pathogens. Since peroxidase is required for random cross-linkage of coniferyl alcohol into lignin [12], increased lignification and perhaps more rapid lignification in response to stress were expected.

High levels of peroxidase expression (up to about 100 times background levels) were obtained in leaf and tuber tissues of transgenic potatoes. In tubers, the foreign peroxidase was glycosylated, presumably at the same molecular sites as in cucumber, as three isoform bands identical in size to those observed in cucumber were observed.

The high peroxidase activity, however, did not confer significant improvement in resistance to pathogens in any of the cultivars. Individual plants with the highest levels of peroxidase activity were no less susceptible to disease. Lignin levels were about the same or slightly lower where transgenic peroxidase levels were high. Possibly the foreign peroxidase interfered with more effective native peroxidase activity by
sequestering cofactors or by other means. Alternatives are that peroxidase expression is not limiting in lignification, or that lignification is not critical to disease response.

The apparent co-suppression of native peroxidase induction on infection complicates analysis, since we do not know the relative potency and specificity of the native peroxidases versus the introduced one. Further, the possibility exists that high peroxidase levels may injure the plant. The transgenic potato plants appeared somewhat short-lived, although generally healthy.

Recent work on plants with altered peroxidase levels has not been free of surprises. We found that very high peroxidase expression does not increase resistance to the tuber pathogen E. carotovora and P. infestans inoculated onto leaves. We also found evidence of possible co-suppression of native peroxidase induction. Lagrimini et al. [11], overexpressing a tobacco anionic peroxidase gene in tomato, found higher lignin levels in several tissues, but resistance to three fungi and one virus did not increase. Sherf et al. [20], examining plants in which the activity of a presumed suberization peroxidase was abolished, found that suberization still occurred at normal rates, which suggests that some other peroxidase may substitute for the missing form.

These authors did not find evidence of co-suppression. Lagrimini also found that tomato and tobacco plants overexpressing tobacco anionic peroxidase wilted sharply on flowering [12, 13]. Our potato plants were harvested without flowering, and wilting was not observed. In tomato, very high levels of soluble phenolic compounds were also observed [21]. The cause of this increase is not understood. In the transgenic tubers, soluble phenolic levels appeared unaltered.

The results of this study indicate that the simple incorporation of a putative defence-associated peroxidase does not necessarily result in the enhancement of resistance. The results, however, do suggest that understanding the function of the peroxidase and determining whether necessary substrates are available for the enzyme are needed before a full evaluation of the role of peroxidase in resistance and why the transgenic potato plants were unchanged in their reaction to infection can be established.

This work was supported by USDA grant 95-31141-1399, the Michigan Potato Industry Commission and the Michigan Agricultural Experiment Station. We are grateful to J. Rasmussen for the plasmids JRF6 and JRF7, to K. Jasztrebski for maintaining greenhouse plants, to Jun Guo for performing the neomycin phosphotransferase assays, and to A. Desjardins and P. S. Bains for providing pathogen strains.

REFERENCES

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