Journal of Chemical Ecology, Vol. 30, No. 7, July 2004 (© 2004)

DIFFERENTIAL ACTIVITY OF PEROXIDASE ISOZYMES IN RESPONSE TO WOUNDING, GYPSY MOTH, AND PLANT HORMONES IN NORTHERN RED OAK (*Quercus rubra* L.)

STEVEN D. ALLISON[1,2] and JACK C. SCHULTZ[1,*]

[1]*Department of Entomology*
Pesticide Research Laboratory
The Pennsylvania State University
University Park, Pennsylvania 16802, USA

(Received January 28, 2003; accepted March 16, 2004)

Abstract—We measured total peroxidase activity and the activities of peroxidase isoforms in leaves of red oak (*Quercus rubra* L.) seedlings exposed to wounding and plant hormones in the greenhouse. Activity of specific peroxidase isoforms was induced differentially by gypsy moth wounding, mechanical wounding, and the wound-associated plant hormone jasmonic acid. Activity of one isoform was enhanced modestly by treatment with salicylate. A study of peroxidase activity in naturally occurring galls elicited on red oak leaves by 12 hymenopteran and dipteran insect species found 16 POD isoforms, 11 of which were differentially induced or suppressed in galls compared with leaves. In both studies, total peroxidase activity as measured spectrophotometrically was not clearly related to activity of these isoforms. These results indicate that red oak seedlings and trees may respond specifically to wounding, particular insects, and plant signals through changes in the activities of individual isozymes.

Key Words—Peroxidase, isozyme, PR protein, *Quercus rubra*, wounding, gypsy moth, jasmonic acid, salicylic acid, galls.

INTRODUCTION

Plant peroxidases (E.C. 1.11.1.7) are heme-containing enzymes whose primary function is to oxidize a variety of hydrogen donors at the expense of hydrogen peroxide. Peroxidase (POD) activity has been correlated with a wide range of

*To whom correspondence should be addressed. E-mail: ujq@psu.edu
[2]Current address: Department of Biological Sciences, Stanford University, Stanford, California 94305-5020, USA.

plant physiological processes, including lignification, suberization, somatic embryogenesis, auxin metabolism, wounding, and disease resistance (Ye et al., 1990; Zimmerlin et al., 1994). PODs are ubiquitous enzymes in plants, often occurring as multiple isoforms; for example, they are encoded by 73 different genes in *Arabidopsis thaliana* (Duroux and Welinder, 2003). Such an abundance of isoforms is consistent with diverse physiological functions for the peroxidase family (Siegel, 1993). POD polymorphisms are often used in taxonomic and population studies.

Plants respond to various environmental challenges with diverse biochemical changes. For example, they commonly elevate concentrations of secondary compounds in response to pathogen infection or insect herbivory (Karban and Baldwin, 1997). Other plant responses include lignification (Vance et al., 1980; Díaz and Merino, 1998) and the production of pathogenesis-related (PR) proteins (Van Loon, 1985). PODs comprise one important class of PR proteins (PR-9) implicated in these "defense responses," in which an important role is to catalyze the formation of phenolic radicals at the expense of H_2O_2 (Gaspar et al., 1985). PODs may also oxidize phenolic monomers to form lignin (Siegel, 1954; Mäder et al., 1980; Grisebach, 1981), function in H_2O_2 production (Elstner and Heupel, 1976; Mäder et al., 1980), and metabolize indole acetic acid (Endo, 1968; Mato et al., 1988). Each plant species typically displays a unique pattern and number of soluble and wall-bound isozymes that may respond differentially to environmental stimuli. Some of the factors known to influence POD isozyme expression are mechanical wounding (Birecka and Miller, 1974; Svalheim and Robertsen, 1990), mite feeding (Bronner et al., 1991), pathogen infection (Lagrimini and Rothstein, 1987; Ye et al., 1990), plant hormones (Ridge and Osborne, 1970; Birecka and Miller, 1974; Neuman et al., 1992), and plant developmental stage (Pao and Morgan, 1988; Biles and Martyn, 1993).

Although POD functions have been well studied in tobacco, horseradish, and other herbaceous species (Birecka and Miller, 1974; Lagrimini and Rothstein, 1987; Kawaoka et al., 1994), activity studies in woody plants are uncommon (Goodin et al., 1993; Dowd et al., 1998a). Since insect attack elicits changes in leaf polyphenols in some oak species (e.g., Rossiter et al., 1988), it is likely that the activities of oak phenolic-oxidizing enzymes and isozymes also change in response to wounding. No studies have examined the enzymatic responses of oak species to herbivores and pathogens, or the role of chemical signaling molecules likely to be involved in these responses.

Responses to herbivores, pathogens, and other environmental stimuli are generally regulated by a network of signal transduction pathways in which jasmonic acid (JA) and salicylic acid (SA) are key signaling molecules (Glazebrook, 2001; Thomma et al., 2001; Kunkel and Brooks, 2002). Wounding and herbivore damage cause rapid increases in JA (Bostock, 1999; Reymond et al., 2000), triggering systemic defenses against herbivores and necrotrophic pathogens. Infection by

biotrophic pathogens can elicit rapid increases in SA (Gaffney et al., 1993; Ryals et al., 1994) and systemic expression of defenses against a range of pathogens.

We assayed POD activity in northern red oak (*Quercus rubra* L.) leaf tissue subsequent to mechanical wounding, wounding by gypsy moth (*Lymantria dispar* L.; Lymantriidae) larvae, and treatment with exogenous JA and SA. We also examined POD activity in galls formed on red oak leaves in response to 12 naturally-occurring insect species. Because red oak produces diverse phenolic substrates (Li and Hsiao, 1975), and because plants are known to express substrate-specific POD isozymes (Calderón et al., 1990), we separated oak PODs by gel electrophoresis and isoelectric focusing and examined elicitation or suppression of individual isoform activities in relation to total POD activity.

METHODS AND MATERIALS

Seedling Oak Growth. Red oak (*Quercus rubra* L.) half-sib seedlings were germinated from acorns collected from a single tree on the Penn State University Park campus in flats containing Metro-Mix 250®growth medium in late June in a glasshouse at 20°C. One week after germination, seedlings were transplanted to 20 × 42-cm pots containing wetted Metro-Mix 250 and 14-14-14 (N-P-K) Osmocote®slow-release fertilizer incorporated at a rate of 550 ml fertilizer beads per 0.085 m^3 of growth medium. Seedlings were watered daily with tap water for 15 min at 7.5 l/hr, and supplemental lighting of 200–350 $\mu E/m^2$ was provided for 13 hr each day.

Treatments. Ten d after transplant, 102 4-wk-old seedlings (each approximately 15 cm in height) were randomly assigned to 1 of 7 treatment or control groups. One group of seedlings served as a control for any POD isozyme induction due to leaf removal or mechanical disturbance. Sample sizes for each treatment and controls ranged from 12 to 15 individual trees. At this age, red oak seedlings have produced one set of 4–5 leaves, all within a day. Leaves were chosen randomly for treatment and/or harvest.

Wounding was accomplished with gypsy moth larvae or mechanically with a hole punch. One to three starved fourth instar gypsy moth larvae were confined manually to a single leaf for the first 12 hr of treatment or until 20–30% of the leaf area was eaten. After removing and flash freezing this leaf, one or more larvae were left on the seedling to continue the insect wounding treatment for 1 wk. Larvae were added or removed over the 1 wk period to accomplish 30–50% leaf area removal. Mechanical wounding with a sterile 6-mm diam hole punch was carried out to mimic insect leaf removal for both the 12-hr and week-long wounding treatments. To prevent the movement of gypsy moth larvae between seedlings, squares of aluminum foil coated in Tanglefoot®were placed around the bases of all 102 seedlings.

Another group of seedlings was sprayed daily (0800 hr) with 5-mM JA in 4% EtOH, or a control solution without JA. Seedlings were removed from the main greenhouse room and sprayed until the JA or JA control solution dripped off the leaves, and allowed to dry for 30 min before being placed back in the main room.

Similarly, additional seedlings were sprayed at the same times with 5-mM SA, consisting of 173-mg SA in 250 ml of 0.005% Triton X-100, or a control solution without SA. The spraying procedure was identical to that of the JA treatment. Both spray regimes were chosen to be consistent with other studies assessing the impact of these signals on enzymatic and metabolic activity and transcript abundance of defense-related genes (e.g., Thaler et al., 1996; Zhang and Baldwin, 1997; Cipollini and Redman, 1999; Moore et al., 2003).

All unsprayed seedlings were jostled and tapped gently on the leaves for a few seconds to account for any seedling response to mechanical disturbance during the JA, SA, or control spraying.

Sampling and Extraction for Enzyme Analysis. Preliminary studies indicated that accumulation of insect-induced polyphenols could be detected after 7–10 d. Therefore, to capture any coordinated changes in POD levels a single treated leaf per seedling was removed at the base of the petiole with scissors 12 hr, 1 wk, and 2 wk (1 wk after the end of treatments) after the start of each treatment; these were flash-frozen in liquid N_2 and stored at -80°C until analysis. Frozen leaf tissue was homogenized in ice-cold extraction buffer with a chilled mortar and pestle at a buffer:tissue ratio of 20:1 (v:w). The extraction buffer contained 0.1-M potassium phosphate, pH 7.0, and 9% (w/v) polyvinylpolypyrrolidone. To break up cellular membranes, 10% (v/v) Triton X-100 was added to the extraction buffer at a rate of 1.6 μl/mg tissue (Jansen et al., 2001). Initially, extracts were centrifuged at 1100 $\times$ g for 15 min at 4°C; however, the viscosity of extracts from older leaves hindered adequate separation of the supernatant and pellet. Thus, samples from older leaves were centrifuged at 15000 $\times$ g for 15 min at 4°C. Densitometric measurements of POD isozyme bands from the same leaf extract exposed to each of the two centrifugation regimes indicated no major differences. Crude extract aliquots were placed into 0.5-ml Eppendorf tubes and frozen at -20°C for later protein assays or used immediately for POD isozyme analysis. These procedures are likely to have extracted primarily soluble PODs. All chemicals were purchased from Sigma Chemical Company, St. Louis, MO, or Bio-Rad Laboratories, Hercules, CA.

Total Peroxidase and Protein Assays. The total soluble POD specific activities of each oak tissue extract were determined by using a microplate reader. To measure total soluble POD, three replicates of 4-μl crude extract were each combined with 196-μl substrate, containing 10-mM guaiacol and 4-mM H_2O_2 in 0.1-M potassium acetate buffer, pH 4.5. The samples were shaken for 10 sec and read at 470 nm for 3 min, and the linear ΔOD/min/mg protein was used as a measure of relative POD activity. Protein content was determined (3$\times$) using the Bio-Rad Detergent-Compatible Protein Assay according to the instructions of the manufacturer.

Isoelectric Focusing. Soluble peroxidase isozymes were separated by using polyacrylamide gel electrophoresis – isoelectric focusing (IEF) with a Model 111 mini-IEF unit (Bio-Rad, Hercules, CA, USA). Gels were cast according to the manufacturer's instructions by using the following components: 50-μl 3/10, 200-μl 8/10, and 700-μl 3/5 ampholyte, 5.05-ml H_2O, 2.0-ml 24.25% acrylamide with 0.75% bis-acrylamide (w/v), and 2.0-ml 25% glycerol (v/v). After moderate degassing, 50 μl of 10% ammonium persulfate (w/v) and 5-μl TEMED were added to initiate polymerization. Preliminary studies indicated that this mixture of components resulted in the least difficulty in casting the gels and best separation of isozymes, which tended to be very acidic or very basic. Standards used were as described in the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

One μl of crude extract was added to each sample well with a Hamilton syringe and allowed to diffuse into the gel for 5 min. Isozymes were separated by stepwise increases in voltage of 100, 200, and 450 V for 15, 15, and 45 min, respectively. Following focusing, gels were soaked in 0.1-M potassium succinate (KSuc), pH 5.5, for 10 min to equilibrate the pH throughout the gel. Peroxidase isozymes were visualized with *o*-dianisidine and H_2O_2 in 0.1-M KSuc, pH 5.5, for 30 min, which was found to be more sensitive and reliable than guaiacol for visualizing POD in gels. The substrate was made by dissolving 8 mg/ml *o*-dianisidine in methanol, combining 2.5 ml of this solution with 97.5-ml KSuc buffer, and adding 44.8-μl 30% H_2O_2.

Immediately after visualization, gels were rinsed 4–6 $\times$ in KSuc buffer, and relative isozyme activities were quantified with a Shimadzu CS-9000U dual-wavelength scanning densitometer. After identifying the linear density range by using a dilution series, each gel lane was scanned at 470 nm (analytical) and 570 nm (reference) with a 0.4 $\times$ 5.0-mm beam. The relative activity of each isozyme was recorded as an integrated absorbance peak at a specific coordinate on the gel lane. Activities are expressed per mg protein. Isozyme bands were compared among different gels and gel lanes by determining their distance relative to a prominent anionic band hereafter called A4.4 and the bottom edge of the 1-cm gel. Isoforms are designated as cationic (C) or anionic (A) and by their isoelectric points (see Figure 1 for naming scheme).

Field Study of Gall PODs. While many galling insects chew host plant tissues, many are thought to suppress or modify normal plant responses to attack (Hartley and Lawton, 1992). We investigated whether such insects would influence POD activities in a manner similar to gypsy moth or artificial wounding. On May 22 and June 3, 1998, galls on red oak leaves formed by the gall wasps (Hymenptera: Cynipidae) *Amphibolips confluens* Harr., *A. inanis* Harr., *A. nubilipennis* Harr., *Andricus pallustris* O.S., *Callirhytis modesta* O.S., *C. pigra* O.S., *and C. tumificus* O.S., and by the gall midges (Diptera: Cecidomyiidae) *Macrodiplosis* spp. *(M. erubescens* O.S., *M. majalis* O.S., *M. niveipila* O.S.), *Polystepha americana* Felt, and an unknown cecidomyiid species were collected from

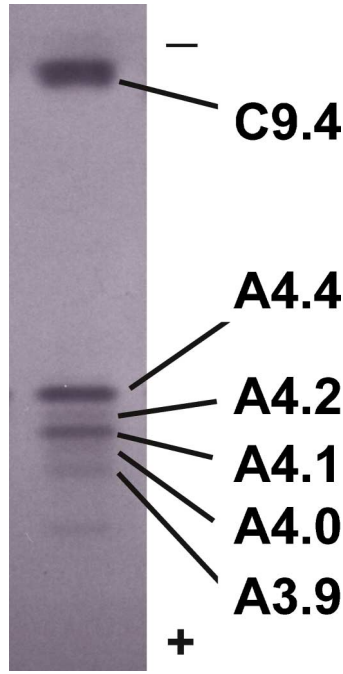

FIG. 1. Example isoelectric focusing gel showing the six most reliably-visualized POD isoforms in greenhouse-grown red oak leaves. Cationic: +, anionic: −.

21 trees in an oak-dominated forest in central Pennsylvania, USA, for peroxidase isozyme analysis. Galls ranged in age from 7 to 15 d; most age differences were between, not within, species. Trees ranged in size from 2 to 25 m, and at least 5 galls were collected from each tree at heights below 3 m. With each gall collected, a nearby ungalled, undamaged leaf of the same position and developmental stage on the same tree was removed at the base of the petiole for comparison. Insects were removed, and all tissues were immediately flash frozen in liquid N_2, held on dry ice, and stored at -20°C until identification and analysis as described above.

Statistical Analyses. Treatment and date effects on isozyme activity, total POD activity, and protein concentration were subjected to a repeated measures analysis of variance by using the mixed procedure of the SAS statistical package (SAS Institute, 1999). A "contrast" statement within the procedure was used to distinguish treatment effects significant at the $\alpha = 0.05$ level. Tukey's studentized range test was used to compare treatment and control means within each date. Paired t tests were used to determine significant differences in isozyme activity and protein content between field-collected red oak galls and leaves.

RESULTS

Total Seedling Protein and Peroxidase. Total protein concentration did not differ among the treatments but dropped slightly after 1 wk (Figure 2). Similarly, total soluble POD activity decreased after 1 wk and then recovered to or exceeded 12 hr levels after 2 wk (Figure 2). Specific contrasts indicated that total POD activity was less in the JA treatment and greater in the SA treatment than in both wounding treatments and the unwounded controls (Figure 2). A lack of difference between JA and SA solvent controls and wound treatments indicates that these were not solvent effects (Figure 2).

Seedling Isozymes. At least 10 POD isozymes were observed in the tissue extracts from red oak seedlings, but only six could be visualized reliably enough within treatments to be considered for statistical analysis (Figure 1). Of the six, one was strongly basic (pI > 9), while the other 5 were strongly acidic (pI < 4.5).

JA treatment and to a lesser extent GM wounding ($P = 0.08$) increased the activity of isozyme A4.1, but there were no significant treatment effects on the cationic isozyme C9.4 or the anionic isozymes A4.2 and A4.0 (Figure 3). All of these isozymes changed over time; activities of C9.4 and A4.1 increased, while activities of A4.2 and A4.0 declined over 2 wk (Figure 3).

Specific contrasts with controls indicated that isozyme A4.4 activity was significantly increased by gypsy moth wounding, mechanical wounding, and JA application, especially after 1 wk (Figure 3). JA treatment caused the greatest response, followed by mechanical wounding and GM wounding. While SA-treated seedlings contained more isozyme A4.4 activity than did untreated controls, SA-treated seedlings did not differ from SA controls, and SA controls (solvent treated) did not differ from untreated controls. The activity of A4.4 increased significantly with time (Figure 3).

The effects of both treatment and date were significant for isozyme A3.9 (Figure 3). Isozyme activity increased strongly over the course of the experiment, and was significantly increased by both gypsy moth and mechanical wounding, but not by JA (Figure 3). Over the entire experiment, there was a marginally significant effect of SA ($P = 0.06$).

Field Gall POD Study. Naturally-occurring galls and leaves exhibited 16 separable isoforms frequently enough for statistical analysis (Table 1). However, not all 21 sampled trees exhibited activity of all 16 isoforms; as few as 8 isoforms could be found in some individuals (data not shown). All galls generally contained significantly enhanced activity (compared with leaves) of cationic isoform C9.4, and significantly reduced activities of cationic isoform C8.2 and anionic isoforms A4.6, A4.4, A4.15, A4.1, A3.7, and A3.9 (Table 2) compared with control leaves on the same trees. Two additional anionic isoforms were suppressed at significance levels $0.10 > P > 0.05$.

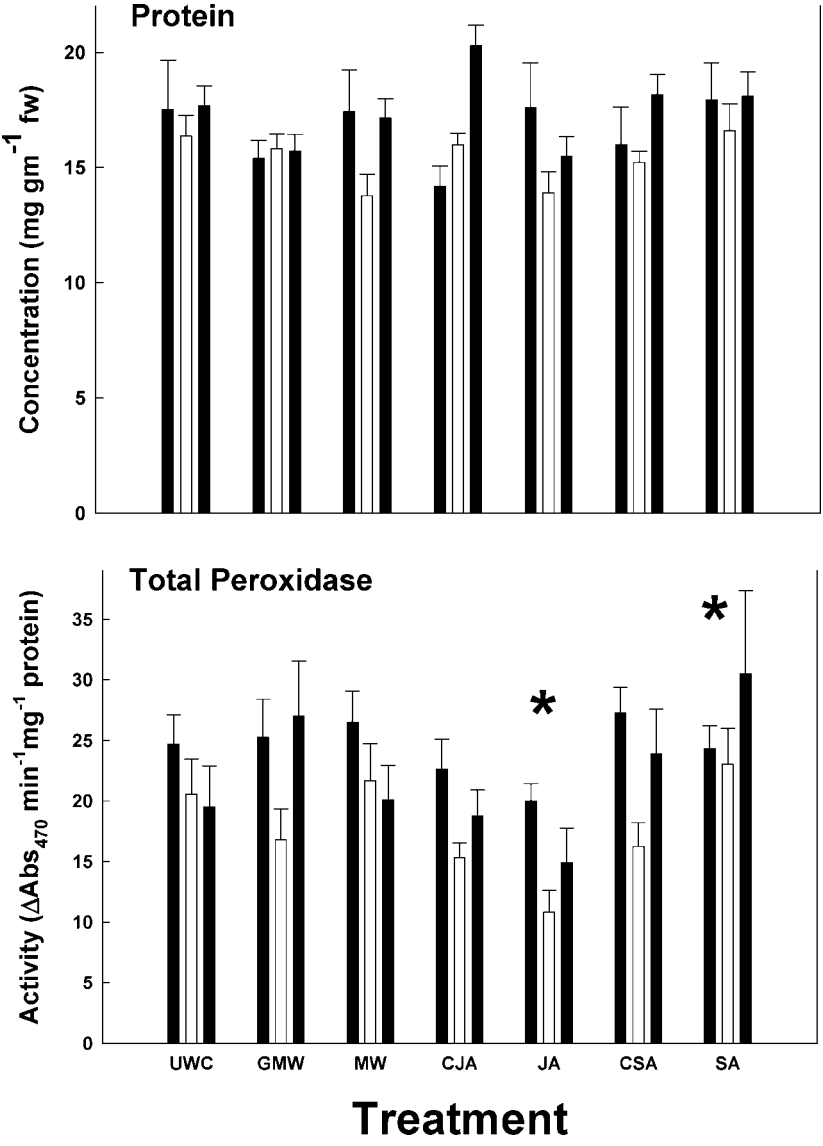

FIG. 2. Means (SE) of relative protein content (top) and total specific POD activity (bottom) of greenhouse-grown red oak leaves after 12 hr, 1 wk, and 2 wk (left to right in each trio of bars) of the following treatments: UWC—unwounded controls, GMW—gypsy moth wounding, MW—mechanical wounding, CJA—jasmonic acid solvent control, JA—jasmonic acid appliction, CSA—salicylic acid solvent control, SA—salicylic acid application. Asterisks indicate significant treatment effects ($P < 0.05$).

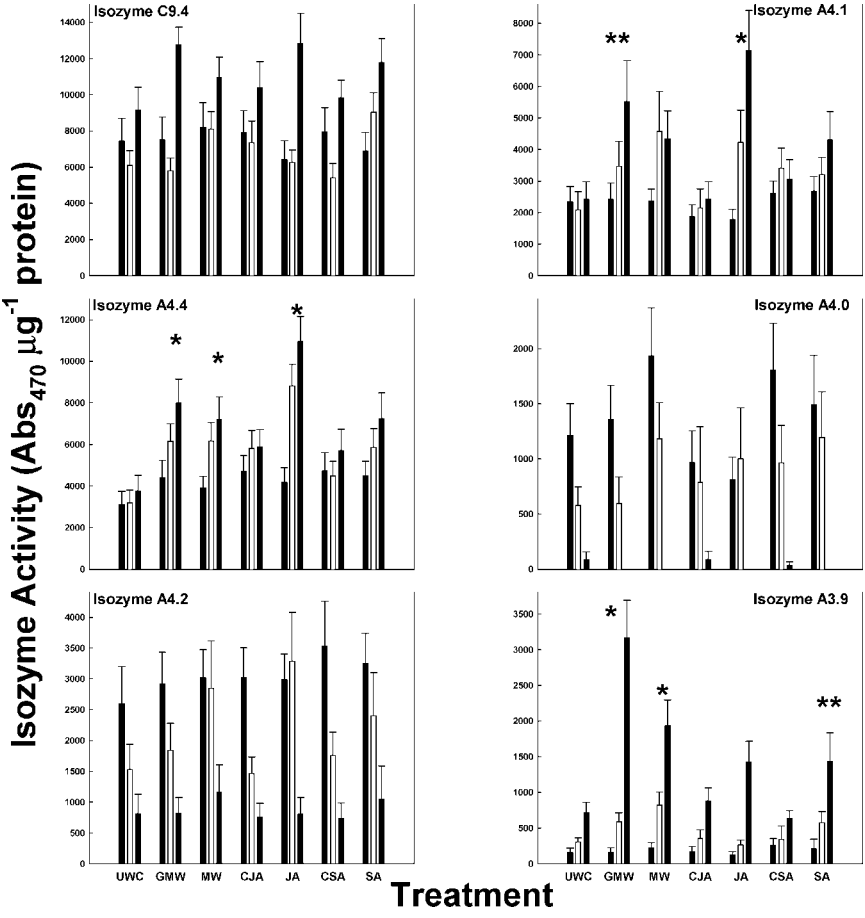

FIG. 3. Means (SE) of the activities of six POD isoforms in greenhouse-grown red oak leaves after 12 hr, 1 wk, and 2 wk of the same treatments indicated in Figure 2. *indicates a significant treatment effect at $P < 0.05$, **indicates $0.05 < P < 0.1$; all isoforms exhibited significant ($P < 0.05$) changes with time.

DISCUSSION

At least 10 POD isozymes were observed in tissue extracts from greenhouse-grown red oak half-siblings and 16 in tissue extracts from field-grown red oaks. These numbers are consistent with a study of POD isozyme variation among and within red oak populations in the eastern US, where 17 total isozymes were noted and 8–12 were found in trees of a given stand (Houston, 1983). This diversity

TABLE 1. POD ISOFORMS FOUND IN FIELD-GROWN RED OAK LEAVES FROM 21 TREES AND THE 6 ISOFORMS QUANTIFIED IN GREENHOUSE STUDY

Field collection isoform identifiction	Isoelectric point	
	Field	Greenhouse
C1	9.87	
C2	9.37	9.4
C3	8.89	
C4	8.21	
A1	5.84	
A2	5.06	
A3	4.82	
A4	4.71	
A5	4.56	
A6	4.37	4.4
A6	4.20	4.2
A7	4.13	4.1
A8	4.06	4.0
A9	3.87	3.9
A10	3.76	
A11	3.68	

of isozymes could provide red oak with flexibility in dealing with common environmental threats such as herbivory, fungal and bacterial pathogen infection, mechanical stress and damage, and air pollutants. In addition, the complex phenolic chemistry of oaks (Li and Hsiao, 1975; Schultz and Baldwin, 1982), may require specific POD isozymes for synthesis or activation (Calderón et al., 1990; Appel, 1993).

In the greenhouse study, activity of red oak POD isozymes varied among half-sib individuals (data not shown) and through time, even within treatments. Variation in isoforms expressed was even greater among field-grown trees. Genetic, biochemical, and developmental factors can all produce this kind of variation within plant species. Loblolly pine cotyledons have as many as 11 isozymes, each of which differs dramatically in activity among seeds of different genotypes (Neuman et al., 1992). Up to 10 isozymes may be found in loblolly needle tissue, but only four of these occur consistently among trees (Snyder and Hamaker, 1978). Flax plants possess four stress-induced and nine constitutive isozymes, some of which arise by differences in posttranslational modification (Fieldes and Gerhardt, 1998). In watermelon seedling stems and cotyledons, novel anionic isozymes appear over the course of development, while others decrease or retain similar levels of activity (Biles and Martyn, 1993). All of these patterns may also be seen in

TABLE 2. DIFFERENCES BETWEEN POD ACTIVITY IN GALL BODIES AND LEAVES FOR 12 WASP AND FLY GALL SPECIES ON RED OAK (*Q. rubra* L.)

Isozyme	# samples with isozyme in leaf or gall[a]	Mean (SE) difference[b]	*T*-statistic	Percent of total isozyme activity[c]
C1	2	−1.24 (1.30)	−1.171	0.2
C2	23	−7.66 (2.58)	−2.961*	26.8
C3	9	−5.92 (5.60)	−1.056	7.1
C4	7	1.23 (0.28)	4.406*	1.2
A1	6	0.40 (0.19)	2.080†	0.3
A2	5	0.49 (0.25)	1.970	0.3
A3	12	0.54 (0.37)	1.476	0.8
A4	8	1.32 (1.38)	0.962	2.9
A5	7	0.58 (0.13)	4.545*	0.3
A6	19	2.62 (0.61)	3.112*	16.8
A7	16	−0.05 (0.44)	−0.092	3.1
A8	22	5.47 (1.55)	3.508*	27.3
A9	16	3.36 (1.46)	2.288*	7.0
A10	22	1.03 (0.52)	1.940†	4.5
A11	6	0.48 (0.17)	2.803*	0.3
A12	15	0.50 (0.14)	3.584*	1.1
Protein content[d]	23	4.24 (1.94)	2.186*	

Note. *T*-statistics were calculated from a paired-difference test for the mean difference: (average host leaf isozyme activity) − (entire gall isozyme activity) for each gall species C = cationic isozyme, A = anionic isozyme.

[a] 23 samples (each sample includes entire gall, galled leaf, and ungalled leaf) were taken from 21 different trees.

[b] Relative absorbance value/unit protein.

[c] The total isozyme activity is a relative measure of the overall prevalence of an isozyme in the entire gall and leaf tissues sampled.

[d] mg protein/g fresh weight.

* $P < 0.05$; † $P < 0.10$.

our northern red oak data. It is likely that we have underestimated the number of significant treatment effects. Many treatments appeared to alter activities of particular isoforms (Figure 3), but individual variation often led to statistical probability values between 0.06 and 0.1.

Activities of all 6 of the red oak isoforms we studied in seedlings appear to be developmentally regulated to some extent (Figure 3). Four increased in activity over time (C9.4, A4.4, A4.1, and A3.9), while the other two decreased (A4.2 and A4.0). Peroxidase isozymes in many other plant species are known to be developmentally regulated (Gaspar et al., 1985), but it is not possible to assign developmental roles to red oak PODs without knowing their specific functions. Some isoforms that increased in activity during our study may have been involved in lignification of the developing leaves (Gaspar et al., 1985; Díaz and Merino, 1998).

Although isozymes A 3.9, A4.4, and A4.1 are all wound-responsive, quantitative differences among them indicate that red oak defensive responses may distinguish among insect wounding, mechanical wounding, and JA treatment. Both GM and mechanical wounding increased the activities of isozymes A4.4 and A3.9, but the response of A3.9 to GM wounding was more pronounced. Isozymes A4.1 and 4.4 responded to both insect wounding and to JA, a signal in the wound response pathway (Zhang and Baldwin, 1997), but these isozymes responded less strongly to mechanical wounding (Figure 3). Isozyme A3.9 failed to change significantly under JA treatment, despite large increases in response to both types of wounding.

Changes in the activities of POD isozymes have been observed in response to pathogen infection and wounding in several plant species (Espelie et al., 1986; Bashan et al., 1987; Lagrimini and Rothstein, 1987; Svalheim and Robertsen, 1990; Ye et al., 1990). In addition, generalized soluble or wall-bound POD activity increases in other plant species in response to wounding (Birecka and Miller, 1974; Kawaoka et al., 1994), pathogens (Bashan et al., 1987), salicylic acid (Rao et al., 1997), and jasmonates (Thaler et al., 1996; Moore et al., 2003). While JA and insect wounding have been found to elevate total soluble POD activity similarly (Choi et al., 1994; Tamari et al., 1995; Zhang and Baldwin, 1997), this study is among the first to suggest that various peroxidase isozymes may be differentially responsive to JA or other signals (Buzi et al., 2004) and the first to find insect-responsive POD isoforms. Our results also indicate that JA does not elicit POD isozymes in exactly the same manner as does gypsy moth herbivory.

Isoform A3.9 activity was enhanced by treatment with SA ($P = 0.055$), a signal more associated with plant defense responses to microbes (Enyedi et al., 1992; Choi et al., 1994; Conti et al., 1996). It is possible, although unlikely, that our trees were incidentally infected by microbes during that particular treatment despite sterilizing the leaf punch, and that A3.9 is both wound- and microbe-responsive. However, no evidence of infection (e.g., necrosis) was observed at any time.

All of the isozymes showing statistically significant increases in response to our greenhouse treatments were anionic. It is possible that cationic isozymes were also induced; additional cationic isozymes are present in red oak and are influenced by galling insects in nature (Allison and Schultz, unpublished), but we were unable to resolve them completely on seedling IEF gels. Although the exact functions of anionic isozymes in red oak are unknown, their induction by wounding is consistent with responses to insects (Felton et al., 1992, Arnason et al., 1994, Dowd, 1994; Dowd et al., 1998a,b). Anionic PODs have been implicated in a variety of leaf-toughening responses, such as lignin biosynthesis (Gaspar et al., 1985; Díaz and Merino, 1998), suberization (Espelie et al., 1986; McDougall, 1993), and cross-linking of cell walls and extensin polymers (Ridge and Osborne, 1970; Everdeen et al., 1988; Bostock and Stermer, 1989). Such toughening processes may decrease the nutritional quality of the plant tissue for herbivores (Bi et al., 1997) as well as

prevent secondary pathogen infection (Vance et al., 1980; Bostock and Stermer, 1989).

Considering the induction in greenhouse seedlings of isozymes A4.4, A4.1, and A3.9, and previous studies of POD wound responsiveness in other plants (e.g., Felton et al., 1994; Bi and Felton, 1995; Bi et al., 1997), we expected total soluble POD activity to increase in response to wounding and JA treatments. Surprisingly, total POD activity was reduced by JA treatment (Figure 1) and was generally unrelated to the isoform values in any clear way. This paradox could be explained by enzyme inhibition in the reaction mixture that is eliminated when isozymes are spatially separated on a gel. More likely, PODs induced by wounding and JA may have greater affinity for *o*-dianisidine than guaiacol, resulting in observable induction only when the former substrate was used on gels. The dip in total POD activity evident after 1 wk in five of seven treatment classes may also be explained by changes in isoforms that were not visualized by our methods; this pattern was not seen in the six measured isoforms (Figure 3).

Activities of various isoforms often differed strongly between the greenhouse seedlings and naturally-occurring trees, and many more isoforms could be identified in field-grown tissues. Cationic isoform C9.4 and anionic A4.1 and A4.2 were unaffected by our seedling treatments, but C9.4 was dramatically suppressed in field-collected galls (compared with matched ungalled leaf tissue), while A4.1 and A4.2 were more active in galls. Suppression and elicitation profiles were characteristic of the insect species involved, although we had to pool results for statistical treatment. Like the differential greenhouse responses to GM, wounding, and JA, this also suggests that red oak POD responses may be unique to the attacking insect. While studies of POD in the context of herbivory have focused on total POD activity (e.g., Cipollini and Redman, 1999; Mayer et al., 2002; Roitto et al., 2003; Traw et al., 2003), the plant pathology literature provides many examples of pathogen-specific POD isoform activity or profiles (Kristensen et al., 1999; Kandan et al., 2002; Tognolli et al., 2002; Maksimov et al., 2003). In some cases, POD isoform function is based on hostplant substrate specificity and the production of specific defenses (Calderón et al., 1990). Transgenic sweetgum (*Liquidambar styraciflua* L.) trees expressing an anionic POD were less palatable to gypsy moth larvae but not all insects (Dowd et al., 1998a). Elevating a single anionic POD in tomato affected some insect herbivores but not others (Dowd et al., 1998b). We suggest that it may be profitable to investigate POD expression profiles in response to specific insects, as well.

We conclude that at least three of the six POD isoforms we observed in greenhouse-grown red oak seedlings respond dynamically and differentially to mechanical wounding, insect wounding, and JA or SA treatments. Furthermore, the activities of these isozymes changed dramatically and differentially over the course of plant development. While plant POD responses to insects are typically measured in terms of total soluble enzyme activity, our results indicate that measurement of

individual isozyme activities may be required to understand fully plant responses to them and other environmental stimuli. Further experiments will be required to determine the exact physiological functions of the POD isozymes present in red oak.

Acknowledgments—We thank H. M. Appel for critical revision of the manuscript, and T. A. Melhuish and S. M. Ketcho for assistance with laboratory work. This research was completed as part of an Honors in Biology thesis in the Schreyer Honors College, PSU, and was supported by NSF DEB-9974067 and REU supplements.

REFERENCES

APPEL, H. M. 1993. Phenolics in ecological interactions: The importance of oxidation. *J. Chem. Ecol.* 19:1521–1552.

ARNASON, J. T., BAUM, B., GALE, J., LAMBERT, J. D. H., BERGVINSON, D., and PHILOGENE, B. J. R. 1994. Variation in resistance of Mexican land races of maize to maize weevil *Sitophilus zeamais*, in relation to taxonomic and biochemical parameters. *Euphytica* 74:227–236.

BASHAN, Y., OKON, Y., and HENIS, Y. 1987. Peroxidase, polyphenol oxidase, and phenols in relation to resistance against *Pseudomonas syringae* pv. *tomato* in tomato plants. *Can. J. Bot.* 65:366–372.

BI, J. L. and FELTON, G. W. 1995. Foliar oxidative stress and insect herbivory: Primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. *J. Chem. Ecol.* 21:1511–1530.

BI, J. L., MURPHY, J. B., and FELTON, G. W. 1997. Antinutritive and oxidative components as mechanisms of induced resistance in cotton to *Helicoverpa zea*. *J. Chem. Ecol.* 23:97–117.

BILES, C. L. and MARTYN, R. D. 1993. Peroxidase, polyphenoloxidase, and shikimate dehydrogenase isozymes in relation to tissue type, maturity and pathogen induction of watermelon seedlings. *Plant Physiol. Biochem.* 31:499–506.

BIRECKA, H. and MILLER, A. 1974. Cell wall and protoplast isoperoxidases in relation to injury, indoleacetic acid, and ethylene effects. *Plant Physiol.* 53:569–574.

BOSTOCK, R. 1999. Signal conflicts and synergies in induced resistance to multiple attackers. *Physiol. Mol. Plant Path.* 55:99–109.

BOSTOCK, R. M. and STERMER, B. A. 1989. Perspectives on wound healing in resistance to pathogens. *Annu. Rev. Phytopath.* 27:343–371.

BRONNER, R., WESTPHAL, E., and DREGER, F. 1991. Enhanced peroxidase activity associated with the hypersensitive response of *Solanum dulcamara* to the gall mite *Aceria cladophthirus* (Acari: Eriophyoidea). *Can. J. Bot.* 69:2192–2196.

BUZI, A., CHILOSI, G., DE SILLO, D., and MAGRO, P. 2004. Induction of resistance in melon to *Didymella bryoniae* and *Sclerotinia sclerotiorum* by seed treatments with acibenzolar-S-methyl and methyl jasmonate but not with salicylic acid. *J. Phytopathol.* 152:34–42.

CALDERÓN, A. A., ANGELES PEDREÑO, M., ROS-BARCELÓ, A., and MUÑOZ, R. 1990. Zymographic screening of plant peroxidase isoenzymes oxidizing 4-hydroxystilbenes. *Electrophoresis* 11:507–508.

CHOI, D., BOSTOCK, R. M., AVDIUSHKO, S., and HILDEBRAND, D. F. 1994. Lipid-derived signals that discriminate wound- and pathogen-response isoprenoid pathways in plants: Methyl jasmonate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3-methylglutaryl-coenzyme A reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. *Proc. Natl. Acad. Sci., U.S.A.* 91:2329–2333.

CIPOLLINI, D. F. and REDMAN, A. M. 1999. Age-dependent effects of jasmonic acid treatment and wind exposure on foliar oxidase activity and insect resistance in tomato. *J. Chem. Ecol*. 25:271–280.

CONTI, G. G., PIANEZZOLA, A., ARNOLDI, A., VIOLINI, G., and MAFFI, D. 1996. Possible involvement of salicylic acid in systemic acquired resistance of *Cucumis sativus* against *Sphaerotheca fuliginea*. *Eur. J. Plant Path*. 102:537–544.

DÍAZ, J. and MERINO, F. 1998. Wound-induced shikimate dehydrogenase and peroxidase related to lignification in pepper (*Capsicum annuum* L.) leaves. *J. Plant Physiol*. 152:51–57.

DOWD, P. F. 1994. Enhanced maize (*Zea mays* L.) pericarp browning: Associations with insect resistance and involvement of oxidizing enzymes. *J. Chem. Ecol*. 20:2497–2523.

DOWD, P. F., LAGRIMINI, L. M., and HERMS, D. A. 1998a. Differential leaf resistance to insects of transgenic sweetgum (*Liquidambar styraciflue*) expressing tobacco anionic peroxidase. *Cell. Mol. Life Sci*. 54:712–720.

DOWD, P. F., LAGRIMINI, L. M., and NELSEN, T. C. 1998b. Relative resistance of transgenic tomato tissues expressing high levels of tobacco anionic peroxidase to different insect species. *Nat. Toxins*. 6:241–249.

DUROUX, L. and WELINDER, K. G. 2003. The peroxidase gene family in plants: A phylogenetic overview. *J. Mol. Evol*. 57:397–407.

ELSTNER, E. F. and HEUPEL, A. 1976. Formation of hydrogen peroxide by isolated cell walls from horseradish (*Armoracia lapathifolia* Gilib.). *Planta* 130:175–180.

ENDO, T. 1968. Indoleacetate oxidase activity of horseradish and other plant peroxidase isozymes. *Plant Cell Physiol*. 9:331–341.

ENYEDI, A. J., YALPANI, N., SILVERMAN, P., and RASKIN, I. 1992. Localization, conjugation, and function of salicylic acid during the hypersensitive reaction to tobacco mosaic virus. *Proc. Natl. Acad. Sci. U.S.A*. 89:2480–2484.

ESPELIE, K. E., FRANCESCHI, V. R., and KOLATTUKUDY, P. E. 1986. Immunocytochemical localization and time course of appearance of an anionic peroxidase associated with suberization in wound-healing potato tuber tissue. *Plant Physiol*. 81:487–495.

EVERDEEN, D. S., KIEFER, S., WILLARD, J. J., MULDOON, E. P., DEY, P. M., LI, X.-B., and LAMPORT, D. T. A. 1988. Enzymic cross-linkage of monomeric extensin precursors *in vitro*. *Plant Physiol*. 87:616–621.

FELTON, G. W., DONATO, K. K., BROADWAY, R. M., and DUFFEY, S. S. 1992. Impact of oxidized plant phenolics on the nutritional quality of dietary protein to a noctuid herbivore. *J. Insect Physiol*. 38:277–285.

FELTON, G. W., SUMMERS, C. B., and MUELLER, A. J. 1994. Oxidative responses in soybean foliage to herbivory by bean leaf beetle and three-cornered alfalfa hopper. *J. Chem. Ecol*. 20:639–650.

FIELDES, M. A. and GERHARDT, K. E. 1998. Apparent differences in the posttranslational modification of peroxidase between flax genotrophs and genotypes and between normal and stress-induced isozymes. *Intern. J. Plant Sci*. 159:504–512.

GAFFNEY, T., FRIEDRICH, L., VERNOOIJ, B., NEGROTTO, D., NYE, G., UKNES, S., WARD, E., KESSMANN, H., and RYALS, J. 1993. Requirement of salicylic-acid for the induction of systematic acquired-resistance. *Science* 261:754–756.

GASPAR, T., PENEL, C., CASTILLO, F. J., and GREPPIN, H. 1985. A two-step control of basic and acidic peroxidases and its significance for growth and development. *Physiol. Plant* 64:418–423.

GLAZEBROOK, J. 2001. Genes controlling expression of defense responses in Arabidopsis—2001 status. *Curr. Opin. Plant Biol* 4:301–308.

GOODIN, M. M., BIGGS, A. R., and CASTLE, A. M. 1993. Changes in levels and isozymes of peroxidase in wounded peach bark. *Fruit Var. J*. 47:185–192.

GRISEBACH, H. 1981. Lignins, *in* pp. 457–478, P. K., Stumpf, and E. E. Conn (eds), The Biochemistry of Plants, Vol. 7. Academic Press, New York.

HARTLEY, S. E. and LAWTON, J. H. 1992. Host–plant manipulation by gall-insects: A test of the nutrition hypothesis. *J. Anim. Ecol.* 61:113–119.

HOUSTON, D. B. 1983. Stand and seed source variation in peroxidase isozymes of *Quercus rubra* L. *Silvae Genet.* 32:59–63.

JANSEN, M. A. K., VAN DEN NOORT, R. A., ADILLAH TAN, M. Y., PRINSEN, E., LAGRIMINI, L. M., and THORNELEY, R. N. F. 2001. Phenol-oxidizing peroxidases contribute to the protection of plants from ultraviolet radiation stress. *Plant Physiol.* 126:1012–1023.

KANDAN, A., COMMARE, R. R., NANDAKUMAR, R., RAMIAH, M., RAGUCHANDER, T., and SAMIYAPPAN, R. 2002. Induction of phenylpropanoid metabolism by *Pseudomonas fluorescens* against tomato spotted wilt virus in tomato. *Folia Microbiol (Praha).* 47:121–129.

KARBAN, R. and BALDWIN, I. T. 1997. *Induced Responses to Herbivory*. University of Chicago Press, Chicago.

KAWAOKA, A., KAWAMOTO, T., OHTA, H., SEKINE, M., TAKANO, M., and SHINMYO, A. 1994. Wound-induced expression of horseradish peroxidase. *Plant Cell Rep.* 13:149–154.

KRISTENSEN, B. K., BLOCH, H., and RASMUSSEN, S. K. 1999. Barley coleoptile peroxidases. Purification, molecular cloning, and induction by pathogens. *Plant Physiol.* 120:501–512.

KUNKEL, B. N. and BROOKS, D. M. 2002. Cross talk between signaling pathways in pathogen defense. *Curr. Opin. Plant Biol.* 5:325–331.

LAGRIMINI, M. L. and ROTHSTEIN, S. 1987. Tissue specificity of tobacco peroxidase isozymes and their induction by wounding and tobacco mosaic virus infection. *Plant Physiol.* 84:438–442.

LI, H.-L. and HSIAO, J.-Y. 1975. A chemosystematic study of the series Laurifoliae of the red oaks: Phenolics of leaves. *Bartonia* 43:25–28.

MÄDER, M., UNGEMACH, J., and SCHLOβ, P. 1980. The role of peroxidase isoenzyme groups of *Nicotiana tabacum* in hydrogen peroxide formation. *Planta* 147:467–470.

MAKSIMOV, I. V., CHEREPANOVA, E. A., and KHAIRULLIN, R. M. 2003. "Chitin-specific" peroxidases in plants. *Biochemistry (Mosc)* 68:111–115.

MATO, M. C., RÚA, M. L., and FERRO, E. 1988. Changes in levels of peroxidases and phenolics during root formation in *Vitis* cultured *in vitro*. *Physiol. Plant* 72:84–88.

MAYER, R. T., INBAR, M., MCKENZIE, C. L., SHATTERS, R., BOROWICZ, V., ALBRECHT, U., POWELL, C. A., and DOOSTDAR, H. 2002. Multitrophic interactions of the silverleaf whitefly, host plants, competing herbivores, and phytopathogens. *Arch. Insect Biochem. Physiol.* 51:151–169.

MCDOUGALL, G. J. 1993. Accumulation of wall-associated peroxidases during wound-induced suberization of flax. *Plant Physiol.* 142:651–656.

MOORE, J. P., PAUL, N. D., WHITTAKER, J. B., and TAYLOR, J. E. 2003. Exogenous jasmonic acid mimics herbivore-induced systemic increase in cell wall bound peroxidase activity and reduction in leaf expansion. *Funct. Ecol.* 17:549–554.

NEUMAN, P. R., FONG, F., NEWTON, R. J., and SEN, S. 1992. Induction of peroxidase isozymes during shoot enhancement by ABA in cotyledon explants of loblolly pine (*Pinus taeda* L.). *J. Plant Physiol.* 139:343–349.

PAO, C.-I. and MORGAN, P. W. 1988. Patterns of total peroxidase activity and isozyme complement during development of selected maturity genotypes of sorghum. *Crop Sci.* 28:531–535.

RAO, M. V., PALIYATH, G. P., ORMROD, D. P., MURR, D. P., and WATKINS, C. B. 1997. Influence of salicylic acid on H_2O_2 production, oxidative stress, and H_2O_2-metabolizing enzymes. *Plant Physiol.* 115:137–149.

REYMOND, P., WEBER, H., DAMOND, M., and FARMER, E. E. 2000. Differential gene expression in response to mechanical wounding and insect feeding Arabidopsis. *Plant Cell* 12:707–719.

RIDGE, I. and OSBORNE, D. J. 1970. Hydroxyproline and peroxidases in cell walls of *Pisum sativum*: Regulation by ethylene. *J. Exp. Bot.* 69:843–856.

ROITTO, M., MARKKOLA, A., JULKUNEN-TIITTO, R., SARJALA, T., RAUTIO, P., KUIKKA, K., and TUOMI, J. 2003. Defoliation-induced responses in peroxidases, phenolics, and polyamines in scots pine (*Pinus sylvestris* L.) needles. *J. Chem. Ecol.* 29:1905–1918.

ROSSITER, M. C., SCHULTZ, J. C., and BALDWIN, I. T. 1988. Relationships among defoliation, red oak phenolics, and gypsy moth growth and reproduction. *Ecology* 69:267–277.

RYALIS, J., UKNES, S., and WARD, E. 1994. Systemic acquired-resistance. *Plant Physiol.* 104:1109–1112.

SAS INSTITUTE. 1999. SAS/STAT User's Guide. SAS Institute, Cary, NC.

SCHULTZ, J. C. and BALDWIN, I. T. 1982. Oak leaf quality declines in response to defoliation by gypsy moth larvae. *Science* 217:149–151.

SIEGEL, B. Z. 1993. Plant peroxidases—an organismic perspective. *Plant Growth Regul*. 12:303–312.

SIEGEL, S. M. 1954. Studies on the biosynthesis of lignins. *Physiol. Plant* 7:41–50.

SNYDER, E. B. and HAMAKER, J. M. 1978. Inheritance of peroxidase isozymes in needles of loblolly and longleaf pines. *Silvae Genet*. 27:125–129.

SVALHEIM, Ø. and ROBERTSEN, B. 1990. Induction of peroxidases in cucumber hypocotyls by wounding and fungal infection. *Physiol. Plant* 78:261–267.

TAMARI, G., BOROCHOV, A., ATZORN, R., and WEISS, D. 1995. Methyl jasmonate induces pigmentation and flavonoid gene expression in petunia corollas: A possible role in wound response. *Physiol. Plant* 94:45–50.

THALER, J., STOUT, M. J., KARBAN, R., and DUFFEY, S. S. 1996. Exogenous jasmonates simulate insect wounding in tomato plants (*Lycopersicon esculentum*) in the laboratory and field. *J. Chem. Ecol*. 22:1767–1781.

THOMMA, B. P. H. J., TIERENS, K. F. M., PENNINCKY, I. A. M., MAUCHMANI, B., BROEKAERT, W. F., and CAMMUE, B. P. A. 2001. Different microorganisms differentially induce Arabidopsis disease response pathways. *Plant. Physiol. Biochem*. 39:673–680.

TOGNOLLI, M., PENEL, C., GREPPIN, H., and SIMON, P. 2002. Analysis and expression of the class III peroxidase large gene family in *Arabidopsis thaliana*. *Gene* 288:129–138.

TRAW, M. B., KIM, J., ENRIGHT, S., CIPOLLINI, D. F., and BERGELSON, J. 2003. Negative cross-talk between salicylate- and jasmonate-mediated pathways in the Wassilewskija ecotype of *Arabidopsis thaliana*. *Mol. Ecol*. 12:1125–1135.

VANCE, C. P., KIRK, T. K., and SHERWOOD, R. T. 1980. Lignification as a mechanism of disease resistance. *Annu. Rev. Phytopath*. 18:259–288.

VANLOON, L. C. 1985. Pathogenesis-related proteins. *Plant Mol. Biol*. 4:111–116.

YE, X. S., PAN, S. Q., and KUC, J. 1990. Activity, isozyme pattern, and cellular localization of peroxidase as related to systemic resistance of tobacco to blue mold (*Peronospora tabacina*) and to tobacco mosaic virus. *Phytopathology* 80:1295–1299.

ZHANG, Z.-P. and BALDWIN, I. T. 1997. Transport of [2-^{14}C]jasmonic acid from leaves to roots mimics wound-induced changes in endogenous jasmonic acid pools in *Nicotiana sylvestris*. *Planta* 203:436–441.

ZIMMERLIN, A., WOJTASZEK, P., and BOLWELL, G. P. 1994. Synthesis of dehydrogenation polymers of ferulic acid with high specificity by a purified cell-wall peroxidase from French bean (*Phaseolus vulgaris* L.). *Biochem. J*. 299:747–753.