Plant Physiol. (1997) 114: 1187–1196

Characterization of Antisense Transformed Plants Deficient in the Tobacco Anionic Peroxidase[1]

L. Mark Lagrimini*, Vicki Gingas, Fernando Finger, Steven Rothstein, and Ting-Ting Y. Liu

Department of Horticulture and Crop Science and Plant Molecular Biology and Biotechnology Program, The Ohio State University, Columbus, Ohio 43210–1096 (L.M.L., V.G., F.F., T.-T.Y.L.); and Department of Molecular Biology and Genetics, University of Guelph, Guelph, Ontario, Canada N1G 2W1 (S.R.)

On the basis of the biological compounds that they metabolize, plant peroxidases have long been implicated in plant growth, cell wall biogenesis, lignification, and host defenses. Transgenic tobacco (*Nicotiana tabacum* L.) plants that underexpress anionic peroxidase were generated using antisense RNA. The antisense RNA was found to be specific for the anionic isoenzyme and highly effective, reducing endogenous transcript levels and total peroxidase activity by as much as 1600-fold. Antisense-transformed plants appeared normal at initial observation; however, growth studies showed that plants with reduced peroxidase activity grow taller and flower sooner than control plants. In contrast, previously transformed plants overproducing anionic peroxidase were shorter and flowered later than controls. Axillary buds were more developed in antisense-transformed plants and less developed in plants overproducing this enzyme. It was found that the lignin content in leaf, stem, and root was unchanged in antisense-transformed plants, which does not support a role for anionic peroxidase in the lignification of secondary xylem vessels. However, studies of wounded tissue show some reduction in wound-induced deposition of lignin-like polymers. The data support a possible role for tobacco anionic peroxidase in host defenses but not without a reduction in growth potential.

There has been considerable interest in plant peroxidases because of their suspected roles in lignification (Grisebach, 1981), introduction of cross-linkages into cell walls (Fry, 1986), metabolism of IAA (Grambow and Langenbeck-Schwich, 1983), disease resistance (Smith and Hammerschmidt, 1988), and insect resistance (Felton and Duffey, 1991). In spite of this interest in peroxidases, little is known of their role in plant metabolism.

Vascular plants have numerous peroxidase isoenzymes (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) with overlapping reactivities toward endogenous substrates. It would be no exaggeration to say that plant peroxidases are among the most studied and yet least understood of plant enzymes. They can carry out one-electron oxidations using H_2O_2 (or oxygen) as an electron acceptor and any number of suitable electron donors, ranging from iodide to macromolecules such as proteins and polysaccharides (Fry, 1986; Dunford, 1991). Individual peroxidase isoenzymes display varying degrees of reactivity toward different substrates; however, there is considerable overlap of activities between isoenzymes. Peroxidases are found in the vacuole and in the cell wall, where they can be either loosely or tightly bound. Some measurable peroxidase activity can be found in most tissues of the plant; however, roots often have the highest activity and largest number of isoenzymes (Lagrimini and Rothstein, 1987). Any plant species may typically have between 10 and 20 peroxidase isoenzymes. Some of these isoenzymes originate from divergent genes, which could differ by more than 50% in peptide sequence (Welinder, 1992). Other isoenzymes may originate from the same gene product and differ only in the carbohydrate moiety (Lagrimini et al., 1990). These limitations make it difficult to develop a model for peroxidase function in plant growth and development; however, the level of complexity can be reduced by selecting one isoenzyme for intensive study.

We have chosen for our purposes the anionic peroxidase from tobacco (*Nicotiana tabacum* L.). This isoenzyme is unique for tobacco in that it is expressed at high levels in the aerial regions of the healthy plant and yet is not found to any appreciable extent in the roots (Lagrimini and Rothstein, 1987). Also, unlike all of the other isoenzymes (approximately 12) in tobacco, anionic peroxidase is not induced by stress (Lagrimini and Rothstein, 1987; Ward et al., 1991). Although the precise role that this enzyme plays in plant growth, development, and stress tolerance remains uncertain, there is significant evidence that this enzyme is involved in lignification (Mäder et al., 1977; Mäder and Füssl, 1982; Lagrimini, 1991). Most significantly, purified tobacco anionic peroxidase is highly active in the polymerization of monolignols such as coniferyl alcohols (Mäder et al., 1977; Mäder and Füssl, 1982), and this isoenzyme is localized in the walls of lignifying vessels (Klotz, 1995). Previously, transgenic tobacco plants that continuously overproduce anionic peroxidase by more than 10-fold were characterized for morphological and physiological abnormalities that could be attributed to this enzyme (Lagrimini et al., 1990). The most noticeable defect in these plants was manifested by the continuous wilting of leaves in the mature plant. It was more recently determined that the severe loss of leaf turgor was a result of the reduced root absorp-

[1] This research was supported in part by a grant from the U.S. Department of Energy (DE-FG02-89ER14004) and by state and federal funds to the Ohio Agricultural Research and Development Center, The Ohio State University, journal article no. 141-96.

* Corresponding author; e-mail lagrimini.1@osu.edu; fax 1–614–292–3505.

Abbreviation: LTGA, lignin thioglycolate.

tive surface, which was a consequence of abnormal root development (Lagrimini et al., 1997). These plants were also characterized for their lignin content and their ability to lignify in response to wounding (Lagrimini, 1991). Lignin levels were consistently higher in transformed plants overexpressing anionic peroxidase. It was also observed that wounded tissue overexpressing anionic peroxidase deposited a lignin-like polymer considerably faster than in nontransformed plants.

In this work transgenic plants have been developed that underproduce tobacco anionic peroxidase using antisense RNA to suppress the activity of the endogenous enzyme. Antisense RNA has been shown to be an effective tool in determining enzyme function (Rothstein and Lagrimini, 1989). We report the characterization of antisense-transformed plants with peroxidase activity reduced by as much as 1600-fold. Anionic peroxidase is shown to have an effect on their overall appearance, growth characteristics, distribution of lignin, and ability to lignify in response to wounding. These results provide further insight into the process of lignification and support a role for tobacco anionic peroxidase in plant growth and host defenses.

MATERIALS AND METHODS

Production of Transgenic Tobacco Plants

The 5′ half of the tobacco (*Nicotiana tabacum* L.) anionic peroxidase cDNA, a 733-bp *Eco*RI-*Acc*I fragment (Lagrimini et al., 1987), was inserted between the cauliflower mosaic virus 35S promoter and terminator (Rothstein et al., 1987). Its orientation was inverted with respect to the promoter to direct the synthesis of antisense RNA. This chimeric antisense peroxidase gene was introduced into the binary plant transformation vector pCib10. The resulting chimeric antisense peroxidase gene, pPOD427, was introduced into the *Escherichia coli*-mobilizing strain S17–1 by DNA transformation. The plasmid pPOD427 was then transferred into *Agrobacterium tumefaciens* strain A136 by conjugation (Rothstein et al., 1987). *A. tumefaciens* strain A136 contains the disarmed helper Ti plasmid pCib542. Exconjugates were selected by their growth on kanamycin. *A. tumefaciens* was used to transform leaf discs of *Nicotiana sylvestris* and *N. tabacum* var Xanthi nc (Horsch et al., 1985). Plants for transformation experiments were grown from seed in the greenhouse with 14-h daily light periods. Young, fully expanded leaves were removed from 8-week-old plants and surface-sterilized briefly with 70% ethanol followed by soaking for 10 min with 10% chlorine bleach. Leaf disc co-cultivation and shoot regeneration were performed as described by Horsch et al. (1985) and Rothstein et al. (1987). Transformed plants were maintained in a medium of Metromix-350 and received continuous feed fertilization (100 μg mL^{-1} nitrogen) in either the greenhouse or controlled environment chambers.

RNA Isolation and Analysis

Total RNA (10 μg) isolated from young, expanded tobacco leaves (Lagrimini et al., 1987) was subjected to agarose-formaldehyde gel electrophoresis (Lehrach et al., 1977) and transferred to a nylon filter (Hybond-N, Amersham). The filters were air-dried briefly and then irradiated RNA-side-up with UV light (256 nm) for 1 min (Khandjian, 1987). Filters were probed with anionic peroxidase cDNA ($pPOD_{3.5}$), washed, and subjected to autoradiography for 48 h at −80°C. RNA size standards were purchased from BRL.

Protein, Enzyme, and Electrophoretic Assays

Tissue (0.5 g) from greenhouse-grown control plants or plants transformed with the chimeric peroxidase antisense RNA gene was analyzed for total peroxidase activity. Tissue was wounded by aseptically dicing it into 5-mm sections and incubating for 48 h at 28°C in the dark on filter paper saturated with Murashige-Skoog medium. Tissue was homogenized with a polytron blender (Brinkmann) in 3 volumes of cold grinding buffer (10 mM sodium phosphate [pH 7.0], and 5 mM sodium metabisulfite). A cleared extract was obtained by centrifugation at 10,000*g* for 20 min. Total peroxidase activity was determined by the increase in A_{470} in 0.28% guaiacol, 0.05 M sodium phosphate buffer (pH 6.0), and 0.3% H_2O_2 (Lagrimini and Rothstein, 1987). Individual peroxidase isoenzymes were identified by IEF. Equivalent aliquots were applied to precast flatbed polyacrylamide IEF gels (LKB Pharmacia, pH 3.5–9.5). After electrophoresis for 1.5 h at 30 W the gels were removed and equilibrated in PBS before being developed for peroxidase activity with 4-chloro-1-naphthol (0.6 mg 4-chloro-1-naphthol/mL PBS, pH 7.0).

Quantification of Lignin and Soluble Phenols

Lignin from plant tissue was first purified from an alcohol-insoluble cell wall fraction by preferentially solubilizing the lignin with thioglycolic acid in a fashion similar to methods used previously (Whitmore, 1978; Bruce and West, 1989). Plant tissue was homogenized with a polytron blender in absolute methanol. Cell walls were pelleted by centrifugation, washed once in methanol, and pelleted again. The cell wall fraction was dried overnight at 60°C. Between 10 and 50 mg of alcohol-insoluble residue was suspended in 5 mL of 2 N HCl and 0.5 mL of thioglycolic acid in screw-cap glass tubes at 100°C. The tubes were place in a 100°C oil bath for 4 h, cooled to room temperature, and the LTGA pellet was collected by centrifugation at 30,000*g* for 10 min at room temperature. The LTGA was washed once with H_2O and solubilized in 5 mL of 0.5 N NaOH for 12 h at 25°C. The extract was cleared by centrifugation (30,000*g*, 10 min) and the supernatant was acidified with 1 mL of concentrated HCl. The LTGA was precipitated at 4°C for 4 h and pelleted by low-speed centrifugation. The precipitate was alkaline-/acid-extracted once more to remove contaminants that absorb in the UV light. The final LTGA precipitate was dried, dissolved in 10 mL of 0.5 N NaOH, and the A_{260} was measured. A standard curve for lignin was generated with dehydroconiferyl alcohol polymerizate

synthesized from coniferyl alcohol and horseradish peroxidase by the method of Kirk et al. (1975).

RESULTS

Selection of Transformed Plants with Suppressed Peroxidase Activity

N. tabacum and *N. sylvestris* plants were transformed with the chimeric antisense peroxidase gene. Ten individual kanamycin-resistant *N. tabacum* and six *N. sylvestris* transformants (T_1) were selected for further growth in the greenhouse. Progenies from these plants were selected through two generations for homozygosity of the transgene (T_3). A sample of leaf tissue was removed from 5-week-old plants for measurement of total peroxidase activity. Relative total peroxidase activities for T_1 and T_3 transformed plants are given in Table I. The data show that total peroxidase activity in the T_1 plants harboring a single copy of the antisense gene is considerably higher than in the T_3 transformed plants containing two transgene copies. The suppression of peroxidase activity in the initial generation is a poor indicator of the efficacy of the antisense RNA in subsequent generations.

Although quite variable, several antisense transformed plants had total peroxidase activity suppressed by as much as 8-fold in leaf tissue. Another indicator of the efficacy of the antisense gene was through the analysis of RNA on blots using anionic peroxidase cDNA as a probe. In one experiment we can confirm that a regenerated plant is transformed and determine the efficacy of the antisense RNA at suppressing the endogenous message. Total leaf RNA from T_1 transformed plants was subjected to northern analysis (Fig. 1). For controls we used RNA from nontransformed plants and plants overexpressing anionic peroxidase (Lagrimini et al., 1990). The predominant RNA band or sense transcript was 1250 nucleotides as predicted. The antisense transcript produced by the foreign gene was 750 nucleotides. All of the initial transformants selected on kanamycin possessed the antisense transcript and produced little or no detectable sense transcript. No further information could be obtained from the blot, since no correlation was seen between the transcript level and total peroxidase activity in the same tissue sample. This was expected because total peroxidase activity reflects the sum activity of all peroxidase isoenzymes. In healthy leaf tissue the anionic isoenzyme accounts for >80% of the total peroxidase activity; however, any number of stresses perceived by the plant could induce the synthesis of other cationic and moderately anionic peroxidases (Lagrimini and Rothstein, 1987).

Figure 1. RNA blots of transformed tobacco plants probed with tobacco anionic peroxidase cDNA. Equivalent amounts of total RNA (10 μg per slot) extracted from T_1 *N. sylvestris* (A) or *N. tabacum* (B) plants regenerated on kanamycin were subjected to denaturing agarose gel electrophoresis. RNA was subsequently blotted onto nylon filters and hybridized to a ^{32}P-labeled anionic peroxidase cDNA probe. Blots were washed and the probe was located and quantified using a phosphor imager. The relative intensity of the bands is indicated on the lower line. The sense anionic peroxidase transcript is 1250 nucleotides and the antisense transcript is 750 nucleotides. Individual antisense transformants are identified with a two-letter code. RNA from a nontransformed plant (NT) and a plant overexpressing anionic peroxidase (OP) were used as a control.

Table I. *Relative total peroxidase activity in the leaves of transformed plants expressing antisense RNA*

Leaf samples were removed from 5-week-old plants. Total peroxidase activity was determined using guaiacol as a substrate.

Plant	Total Peroxidase Activity	
	T_1	T_3
	% of untransformed plant	
N. tabacum		
AB	83	34
AD	111	13
AF	83	37
GG	83	20
CC	67	53
EE	17	13
KK	91	53
QQ	63	45
YY	125	59
ZZ	333	100
N. sylvestris		
AB	125	36
AC	125	13
AD	143	16
AG	167	31
YY	71	32
RR	38	13

To more accurately describe the expression of anionic peroxidase and the efficacy of the antisense RNA, we dissected the stems of transformed and nontransformed plants into tissue components and measured total peroxidase activity. The results are shown in Table II. In the nontransformed plant, peroxidase activity was lowest in the secondary xylem (0.75 unit/mg protein) and highest in the stem epidermis (193 units/mg protein). Antisense RNA suppression of peroxidase activity was most effective in those tissues with the highest peroxidase activity (193.57–0.12 units/mg protein in the epidermis) and least effective in the secondary xylem (0.75–0.46 units/mg protein). It was surprising to see such low peroxidase activity in the xylem, where the enzyme would function in lignification.

Morphological and Physiological Characterization of Transformed Plants

Possibly the most striking phenotype in plants with suppressed peroxidase activity was their similarity to non-

Table II. *Total peroxidase activity[a] in various stem tissues from the N. sylvestris plant AC expressing antisense RNA to tobacco anionic peroxidase*

Nontransformed/ Tissue	Peroxidase Activity		Nontransformed
	Nontransformed	AC	
	unit/mg protein		*%*
Pith, inner	17.26	0.34[b]	1.97
Primary xylem/interior phloem	4.09	2.90	70.90
Secondary xylem	0.75	0.46	61.33
Cortex/exterior phloem	11.24	5.21[b]	46.35
Epidermis	193.57	0.12[b]	0.06

[a] Total peroxidase activity was determined by guaiacol assay; 1 unit = 1 ΔA_{470}/min. [b] Statistically different from controls.

transformed plants. Casual observation of the antisense-transformed plant revealed no noticeable differences. This is in sharp contrast to transgenic plants, which overexpress anionic peroxidase (Lagrimini et al., 1990). Differences are only seen after a close comparison of the antisense plant with nontransformed plants and with plants overproducing anionic peroxidase. Seed from the three genotypes was sown at the same time and grown in the greenhouse under identical conditions. Both *N. tabacum* and *N. sylvestris* antisense-transformed plants were compared for both growth rate and gross morphology. Representative *N. sylvestris* plants are pictured at the first appearance of flowers (Fig. 2A). Those plants with suppressed peroxidase activity consistently bolted and flowered 3 d before the nontransformed plants. Plants overproducing peroxidase were considerably smaller and flowered 3 d after the nontransformed plants (Fig. 2A).

Similar differences were seen in antisense-transformed *N. tabacum* plants. Figure 2B shows differences in the growth rate, as demonstrated by plant height. Twelve weeks after sowing (approximately 1 week before flowering), the heights of *N. tabacum* plants were measured (Table III). Growth experiments were repeated on three occasions, and on each occasion those plants underproducing peroxidase were significantly taller than the nontransformed plants, and those overproducing peroxidase were significantly shorter than the nontransformed plants. Variations in growth and cell expansion were also detected at the cellular level. An example of this is seen in leaf thickness. Leaf cross-sections were prepared from equivalent regions of the first fully expanded leaf of transgenic *N. sylvestris* plants (Fig. 2C). Leaf thickness increased by an average 17% in underproducing plants and decreased by 30% in overproducing plants (Table IV). Differences in growth rate and vigor could also be seen in the germinating seed. Approximately 200 seeds from each of the three genotypes of *N. sylvestris* were germinated in liquid Murashige-Skoog medium. Representative seedlings at 5 d after imbibition are shown in Figure 2D. At that time >95% of the nontransformed seed germinated. The same was observed for the seeds that underexpress peroxidase; however, the cotyledons were more developed. However, only 50% of the seeds that overexpress anionic peroxidase had broken free from the seed coat (Fig. 2D).

The growth of axillary shoots was also affected by peroxidase expression. Transformed *N. sylvestris* plants were

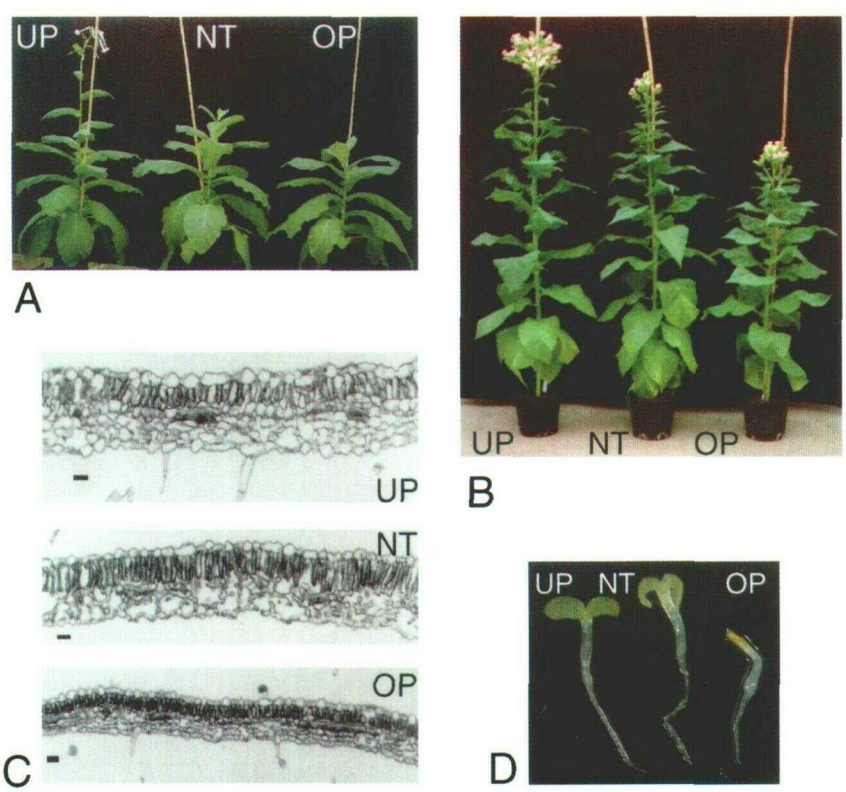

Figure 2. Growth characteristics of transformed plants with altered peroxidase expression. Tobacco seeds (>25 repetitions) either underproducing (UP) or overproducing (OP) anionic peroxidase were sown simultaneously with nontransformed plant material (NT) and grown in the greenhouse at 28°C with 16 h of light. Representative plants are shown at the time of flowering: A, *N. sylvestris;* B, *N. tabacum;* and C, Representative leaf cross-sections from transformed tobacco plants either underexpressing or overexpressing tobacco anionic peroxidase. Tissue samples were taken from the first fully expanded leaf (leaf no. 10 counting from the base of the plant) from 8-week-old *N. sylvestris* plants. For consistency, the region of the leaf used for sampling was midway between the petiole and tip and midway between the midvein and outer leaf edge. Leaf samples were fixed and imbedded in paraffin, and 13-μm sections were prepared with a microtome. The black bar indicates 100 μm. D, Six-day-old *N. sylvestris* seedlings germinated in liquid Murashige-Skoog medium at 28°C in the light.

Table III. *Average height for 12-week-old greenhouse-grown N. tabacum plants*

Three experiments were conducted at different times of the year. A different letter superscript beside a value represents a significant difference of means according to Duncan's Multiple Range test, ($P \leq 0.05$). Data are means ± SE.

Genotype	Plant Height		
	Experiment 1 ($n = 57$)	Experiment 2 ($n = 58$)	Experiment 3 ($n = 34$)
		cm	
Underproducing	130.8 ± 12.3[a]	115 ± 20.1[a]	102.15 ± 8.8[a]
Nontransformed	107.6 ± 8.3[b]	100.9 ± 13.11[b]	90.33 ± 6.42[b]
Overproducing	92.5 ± 9.8[c]	95.31 ± 11.94[c]	81.51 ± 15.04[c]

grown in the greenhouse at 200 μg mL^{-1} nitrogen (twice the typical rate) to promote the vegetative growth of the plant. Under this fertilization regimen we consistently observed more highly developed axillary buds in the transformed plants underproducing peroxidase. In contrast, the growth of the axillary buds was suppressed in the transformed plants overproducing peroxidase. Figure 3A shows the eighth node of equivalent *N. sylvestris* plants with altered peroxidase expression. A comparison of the axillary inflorescences of flowering plants is pictured in Figure 3B. Anionic peroxidase demonstrated a negative effect on the growth of axillary shoots.

Distribution of Lignin in Antisense-Transformed Plants

There is considerable evidence indicating that peroxidases and, more specifically, tobacco anionic peroxidase, catalyze the polymerization of monolignols into lignin (Mäder et al., 1977; Mäder and Füssl, 1982; Lagrimini, 1991; Lagrimini et al., 1993). If the anionic isoenzyme is involved in this reaction, there should be a correlation between the lignin content and peroxidase activity observed. Therefore, we determined the lignin content in various tissues from nontransformed and transformed plants either over- or underexpressing tobacco anionic peroxidase. The LTGA assay used here is very sensitive and reproducible in soft tissues; however, it is not entirely specific for lignin. For this reason, a limited number of leaf samples from transgenic plants were verified for lignin composition by thioacidolysis (Chabbert et al., 1992). Lignin levels by thioacidolysis showed similar trends as with the LTGA assay; however, a more precise definition of what we are reporting is lignin-like polymers.

Table V shows the lignin content determined for young, woody stem tissue from several independent transformants of *N. tabacum* with reduced peroxidase activity. The lignin content in the woody stems of transformed lines underproducing peroxidase was the same or somewhat higher than in the nontransformed plants. Mature root tissue was also assessed for lignin content. As with the stems, lignin content did not change in root tissue with reduced peroxidase activity. A similar result was seen in leaf tissue. As expected, leaf tissue had significantly less lignin than the other tissues (<1%). Mature leaves showed a slightly elevated lignin content, as was seen in woody stem tissue.

Wound Response for Transgenic Pith Tissue

A large number of peroxidase isoenzymes in tobacco are known to be induced by wounding; however, the anionic isoenzyme is not affected by wounding (Lagrimini and Rothstein, 1987). Nevertheless, previous results indicated that the overexpression of anionic peroxidase in tobacco and tomato had increased the rate in which lignin was deposited in response to wounding (Lagrimini, 1991; Lagrimini et al., 1993). We were curious to determine whether the antisense suppression of anionic peroxidase would have an effect on the wound-induced production of soluble phenolics and lignin-like polymers. First, it was necessary to show that the antisense RNA to the anionic peroxidase message was specific to this isoenzyme and had no effect on wound-induced peroxidase isoenzymes; therefore, leaf tissue homogenates were prepared from the antisense-transformed plant and the nontransformed *N. sylvestris* plant. Parallel tissue samples were wounded by crushing followed by a 48-h incubation. Wounded tissues were used to visualize peroxidase isoenzymes not observed in healthy leaf tissues (Lagrimini and Rothstein, 1987). Peroxidase isoenzymes were separated by IEF acrylamide gel electrophoresis and visualized by development for peroxidase activity (Fig. 4). It can be seen from this gel that the anionic isoenzyme (pI 3.75) was reduced in the antisense-transformed plant (Fig. 4, lanes 3 and 4) and that other peroxidase isoenzymes were unaffected by the antisense RNA. With confirmation that the antisense RNA was specific for the anionic isoenzyme, pith tissue was aseptically removed from control and antisense-transformed plants, and the wounded tissue was incubated at 28°C in a moist, dark chamber.

Table IV. *Average leaf thickness for equivalent tissues of greenhouse-grown N. sylvestris plants*

A different letter superscript beside a value represents a significant difference of means according to Duncan's Multiple Range test. Data are means ± SE ($n = 25$, $P \leq 0.05$).

Genotype	Leaf Thickness
	μm
Underproducing	618 ± 9.6[a]
Nontransformed	512 ± 5.8[b]
Overproducing	357 ± 16.0[c]

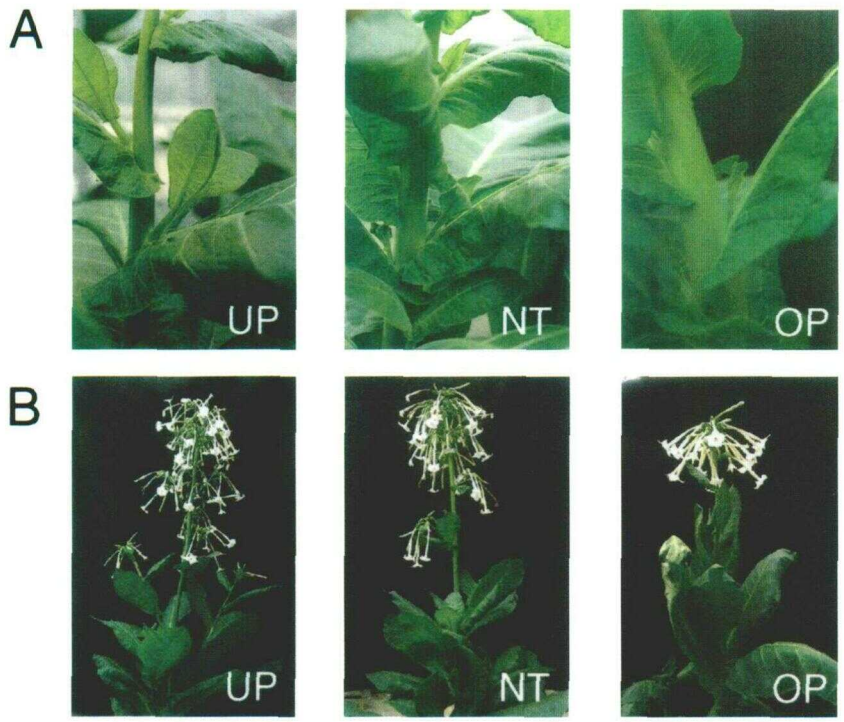

Figure 3. Axillary shoot growth characteristics in transformed *N. sylvestris* plants either underproducing (UP) or overproducing (OP) anionic peroxidase. Transformed plants and nontransformed plants (NT) were grown under standard greenhouse conditions (see "Materials and Methods") with the exception that fertilization was at 200 μg mL^{-1} nitrogen (twice the normal rate). This rate accelerates axillary shoot growth in tobacco. A, Axillary shoots at the 12th node from the base of the plant at the initiation of flowering. B, Comparison of floral tillers at full flowering.

Figure 5A shows the increase in total peroxidase activity in wounded pith tissue for nontransformed plants and those transformed with the antisense anionic peroxidase gene. No measurable effect on total peroxidase activity was observed until 5 d after wounding, at which time the antisense-transformed plant showed 30% lower enzymatic activity. The same samples were subjected to IEF gel electrophoresis and stained for peroxidase activity. Figure 5B shows that the antisense RNA had no effect on the activity of a wound-inducible isoenzyme (pI 8.9). This figure also shows that wounding had no effect on the activity of the anionic isoenzyme. Soluble phenols accumulated in both plants in response to wounding, although transformed plants had lower initial levels (Fig. 5C). The accumulation of lignin-like polymers in response to wounding was shown previously to be accelerated in plants overexpressing anionic peroxidase (Lagrimini, 1991). Figure 5D shows

Table V. *Lignin content for stem, root, and leaf tissue from transformed and nontransformed N. tabacum plants that underexpress tobacco anionic peroxidase*

Plant	Tissue Source	POD[a]	Lignin[b]
		units g^{-1} fresh wt	*% dry wt*
NT1	Young stem	429.6	5.36 ± 0.06
NT2	Young stem	335.6	5.80 ± 0.03
EE1	Young stem	2.9	6.74 ± 0.02
EE2	Young stem	4.6	6.22 ± 0.11
GG1	Young stem	7.4	5.89 ± 0.03
GG2	Young stem	13.9	6.06 ± 0.02
NT1	Mature root	144.1	3.41 ± 0.03
NT2	Mature root	216.3	3.75 ± 0.09
EE1	Mature root	140.2	2.96 ± 0.12
EE2	Mature root	115.5	3.52 ± 0.05
GG1	Mature root	69.7	2.30 ± 0.23
GG2	Mature root	78.6	4.22 ± 0.06
NT1	Mature leaf	109.4	0.63 ± 0.01
NT2	Mature leaf	131.1	0.66 ± 0.01
EE1	Mature leaf	23.2	0.84 ± 0.01
EE2	Mature leaf	28.1	0.70 ± 0.01
GG1	Mature leaf	23.4	0.70 ± 0.01
GG2	Mature leaf	19.1	0.83 ± 0.02

[a] Total peroxidase activity determined by guaiacol assay; 1 unit = 1 ΔA_{470}/min. [b] Lignin content as determined by LTGA is mean ± SE (n = 4).

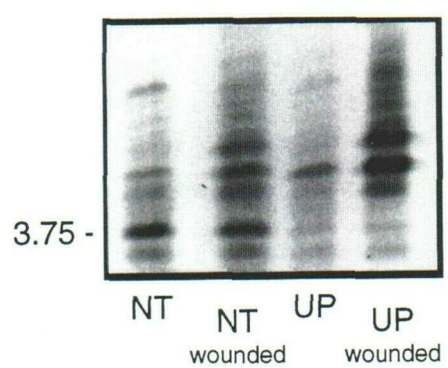

Figure 4. Peroxidase isoenzymes from control and transformed tobacco plants. Enzymes were extracted from *N. sylvestris* pith tissue either immediately removed from the plant or 48 h after the tissue was wounded. Peroxidase isoenzymes were separated on acrylamide gels by IEF and visualized with 4-chloro-1-naphthol. Lanes 1 and 2 are peroxidase isoenzymes from nontransformed tissue, and lanes 3 and 4 are isoenzymes from the antisense-transformed plant designated AC10. Lanes 1 and 3 are isoenzymes from unwounded tissue and lanes 2 and 4 are from wounded tissue. Labels are as in Figure 3.

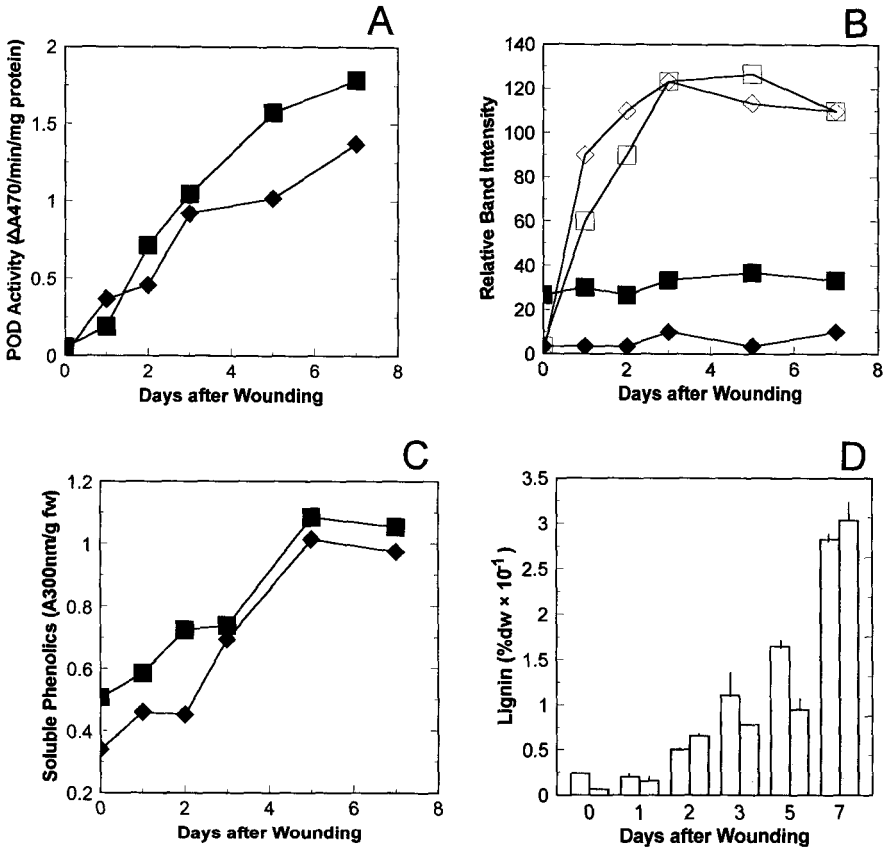

Figure 5. Comparison of the wound response between nontransformed and transformed tobacco plants underexpressing anionic peroxidase. Pith tissue was aseptically removed from mature *N. sylvestris* plants, cut into 5-mm discs, and incubated on moist filter paper in the dark for 48 h at 28°C. A, Total peroxidase in wounded pith tissue for nontransformed (■) and underproducing (◆) plants. B, Relative activity of anionic peroxidase (pI 3.5; filled symbols) and a wound-inducible peroxidase (pI 8.9; open symbols) in wounded nontransformed (■, □) and underproducing (◆, ◇) pith tissue. Peroxidase isoenzymes were separated by IEF and developed for peroxidase activity with 4-chloro-1-naphthol, and the relative band intensity was determined by densitometry. C, Increase in total soluble phenolics in wounded nontransformed (■) and underproducing (◆) pith tissue, as determined by A_{300}. fw, Fresh weight. D, Lignin content in wounded pith tissue of nontransformed (open bars) and underproducing (shaded bars) plants. Lignin was determined by thioglycolic acid extraction and is presented as the percentage of dry weight (n = 4).

the increase in lignin in response to wounding. Wound-induced deposition of lignin-like polymers was reduced at d 3 and 5 in the antisense-transformed plant, and by d 7 the lignin content was similar to nontransformed tissue.

DISCUSSION

Modified Plant Growth in Tobacco Plants with Genetically Altered Anionic Peroxidase Expression

The genetic manipulation of anionic peroxidase gene expression results in observable changes in plant growth and development. These changes are consistent with a role for this enzyme in modifying one or more of the fundamental signaling pathways in controlling plant growth. This is an ambitious task for a cell wall-associated enzyme such as anionic peroxidase; however, many of the biologically active compounds in the plant do pass through the wall and coincidentally are metabolized by peroxidase. These compounds include H_2O_2 (Tenhaken et al., 1995), IAA (Gazaryan and Lagrimini, 1996; Gazaryan et al., 1996), dehydrodiconiferyl alcohol glucosides (Teutonico et al., 1991), and arachidonic acid (Garner, 1984). The altered activity of anionic peroxidase in transgenic plants could dramatically affect the concentration and mobility of any of these biologically active substances. For purposes of discussion, we would like to focus on the role of tobacco anionic peroxidase in the metabolism of IAA. This is done for two reasons. First, the changes in growth observed in transformed plants with modified peroxidase expression are consistent with a role in IAA metabolism, and second, much is known about the oxidative decarboxylation of IAA in vitro by peroxidase.

Approximately 85% of the IAA metabolized in corn plants can be accounted for through nondecarboxylative processes such as conjugation (Cohen and Bandurski, 1978; Epstein et al., 1980). If we assume that these figures would be the same for tobacco, as much as 15% of the auxin could be metabolized via peroxidase. If this were the case, the antisense suppression of anionic peroxidase would lead to the accumulation of IAA, and the overproduction of the enzyme would result in lower IAA levels. Measurements of IAA levels in transformed tobacco tissues have not yet revealed a significant effect of peroxidase activity (H.C. Wang and L.M. Lograimini, unpublished results); however, growth and developmental patterns may be mediated by the ratio of auxin to cytokinin, as opposed to absolute amounts of each hormone (Romano et al., 1991). In lieu of quantitative evidence, it is appropriate to review the phenotypes observed in transgenic plants with altered peroxidase activity and plants with altered auxin or cytokinin biosynthesis. Transformed plants underproducing anionic peroxidase grow taller and flower sooner than nontransformed plants. The leaf lamina structure is less organized with larger cells, and they show more vigorous growth of axillary shoots. Transformed plants overproducing anionic peroxidase grow slower and flower later than nontrans-

formed plants. Their leaf lamina is thinner, with smaller cells, and there is slower growth of the axillary buds. These plants also have a smaller, less branched root system (Lagrimini et al., 1997). Overall, under optimal growth conditions, anionic peroxidase has a negative influence on plant growth and vigor.

Many of the phenotypes observed in transgenic plants with altered anionic peroxidase expression are reminiscent of plants with altered phytohormone metabolism. Most interesting are those plants that overproduce IAA (Sitbon et al., 1992), are unable to accumulate IAA (Romano et al., 1991), or overproduce cytokinin (Hewelt et al., 1994). These phenotypes are summarized in Table VI. Several conclusions can be drawn. First, the data from the three lines with altered auxin/cytokinin metabolism do not always fit with predicted roles for auxin/cytokinin action. For example, time to flowering is increased in all three transformed lines. Second, other phenotypes observed, such as axillary bud formation, fit perfectly with the prescribed roles for auxin and cytokinin and a role for anionic peroxidase in IAA metabolism. Actually, based on phenotypes, there is a better correlation seen for anionic peroxidase affecting the ratio of auxin to cytokinin than that which is seen in plants with altered auxin and cytokinin metabolism. We are currently crossing our anionic peroxidase-overproducing and -underproducing plants with a tobacco line that overproduces IAA (Sitbon et al., 1992) to look for a reversion of the phenotypes.

Roles for Tobacco Anionic Peroxidase in Lignification and Defense

Antisense RNA has been demonstrated time and again to be an effective method to silence gene expression in plants (Rothstein and Lagrimini, 1989). In most cases the predicted phenotype is observed in transformed plants harboring the antisense gene, e.g. virus resistance (Powell et al., 1989), delayed senescence (Picton et al., 1993), and altered partitioning of sugar (Kuipers et al., 1995). However, there are some notable exceptions in which the antisense RNA suppression of gene expression produces unexpected results. Rodermel et al. (1988) showed that the antisense suppression of Rubisco had no effect on plant growth. Later, it was shown that the antisense leaves accumulated normal levels of chlorophyll and managed to maintain optimal photosynthetic rates, regardless of the low activity of Rubisco (Jiang and Rodermel, 1995). Similarly, the complete antisense RNA suppression of the chlorophyll *a/b*-binding protein in transgenic tobacco resulted in no change in chlorophyll content or the assembly of PSII (Flachmann and Kuehlbrandt, 1995). Sherf et al. (1993) reported that antisense RNA suppression of a suberization-associated peroxidase in transgenic tomato had no effect on wound-induced suberization. In the latter case it was hypothesized that another peroxidase isoenzyme was able to substitute for the missing enzyme. Sometimes the utility of antisense RNA is realized when the predicted outcome is not achieved. This is keenly demonstrated when antisense RNA is used to suppress β-1,3-glucanase activity. This enzyme has long been considered important in the defense against disease; however, recent results from antisense transformation experiments suggest that the enzyme may assist in viral infection (Beffa et al., 1996).

It was predicted that antisense RNA suppression of tobacco anionic peroxidase would result in a measurable decrease in lignification. This was based on previous studies of the purified enzyme (Mäder et al., 1977) and the result of overexpression in transformed tobacco and tomato plants (Lagrimini, 1991; Lagrimini et al., 1993). Although the suppression of anionic peroxidase activity by antisense RNA in transformed plants was effective, there was no significant effect on lignification. There are four possible explanations for this result: (a) Residual anionic peroxidase activity is sufficient for lignification. Antisense RNA is not 100% effective in suppressing anionic peroxidase activity (Table II; Fig. 4). It is possible that the enzyme is produced in much larger quantities than is required for lignification. This explanation is always a possibility in antisense-transformation experiments that do not result in the predicted outcome. However, in this case there were numerous phenotypes observed in antisense-transformed plants, indicating its ability to effect nonlignification processes. (b) The antisense suppression of anionic peroxidase affects IAA metabolism, which, in turn, induces the phenylpropanoid pathway and promotes lignification. The effect of peroxidase expression on plant growth and development presented here is suggestive of a role in hormone metabolism, likely IAA. The depletion of this enzyme may slow the metabolism of IAA, thus leading to higher endogenous levels of this hormone. Auxin has been shown previously to induce the phenylpropanoid pathway and promote lignification and vessel formation (Aloni, 1987). The

Table VI. *Phenotypes observed in transgenic tobacco plants with altered anionic peroxidase expression and altered auxin or cytokinin metabolism*

	Peroxidase		IAA		Cytokinin, Overproducing[c]
	Overproducing	Underproducing	Overproducing[a]	Underproducing[b]	
Growth rate	Decreased	Increased	nd[d]	Decreased	Decreased
Height	Shorter	Taller	Shorter	Same	Shorter
Time to flower	Longer	Shorter	Longer	Longer	Longer
Axillary bud growth	Decreased	Increased	Decreased	Increased	Increased
Root branching	Decreased	Increased[e]	Increased	Decreased	Decreased
Lignin content	Increased	Same	Increased	Decreased	nd
Chronic wilting	Yes	—	—	—	Yes

[a] Sitbon et al., 1992. [b] Romano et al., 1991. [c] Hewelt et al., 1994. [d] nd, Not done. [e] Data not shown.

lower levels of the enzyme may be overwhelmed by activation of the overall pathway, thus negating any effect of the antisense RNA. This theory could be supported if the activity of the enzymes participating in phenylpropanoid metabolism is higher in antisense-transformed plants. (c) A peroxidase isoenzyme other than anionic peroxidase or a laccase catalyzes the polymerization of monolignols in the absence of anionic peroxidase. The nature of the enzyme involved in the free radical polymerization of monolignols into the lignin polymer is unclear. Most plant peroxidases are capable of forming a lignin-like polymer in the test tube, and this activity in vitro was used to support a role for peroxidase in lignification (Harkin and Obst, 1973). More recently, a similar activity in vitro was shown for plant laccases (Dean and Eriksson, 1994). (d) Anionic peroxidase is not responsible for the deposition of lignin in vessels. As just mentioned, other peroxidases or laccases can synthesize lignin in vitro. It is very possible that one of these enzymes, as opposed to anionic peroxidase, catalyzes the deposition of the lignin polymer. This is supported by the absence of peroxidase enzymatic activity or anionic peroxidase gene expression in highly lignified tissues (Table II; Klotz, 1995). The woody secondary xylem of the tobacco stem is the most highly lignified tissue in the plant (>6% lignin); however, this tissue has nearly undetectable peroxidase activity (<1 unit/mg protein).

Regardless of which explanation is correct, there is compelling evidence that anionic peroxidase can have a limited role in lignification. The overexpression of this enzyme consistently results in higher lignin content, even in those tissues that do not normally lignify (Lagrimini, 1991; Lagrimini et al., 1993). Perhaps the primary role for the anionic peroxidase of tobacco is in host defense and stress-induced lignification. Thus far, there are two lines of evidence supporting this theory. First, by far the highest levels of this enzyme are found in the epidermis (Table II) and the trichomes (Klotz, 1995). The epidermis is the first line of defense, and this tissue is not normally lignified. In the absence of any other function for the enzyme in the epidermis, a defensive role seems reasonable. Second, more direct evidence for a defensive role can be seen in transgenic plants subjected to insect attack. Transformed tobacco, tomato, and sweet gum overproducing tobacco anionic peroxidase were all found to exhibit greater resistance to insect attack (Dowd and Lagrimini, 1997). At the same time, antisense-transformed tobacco plants were found to be more susceptible to insect damage. Together, these results support a role for tobacco anionic peroxidase in host defenses. It is our belief that the primary role for the tobacco anionic peroxidase is defense; however, it is not without cost, as measured by reduced growth potential.

ACKNOWLEDGMENTS

We would like to thank Hao-Ching Wang, Ruth Fulton, and Monica Kmetz-Gonzales for their assistance with the plant growth experiments.

Received January 31, 1997; accepted April 26 1997.
Copyright Clearance Center: 0032–0889/97/114/1187/10.

LITERATURE CITED

Aloni R (1987) Differentiation of vascular tissues. Annu Rev Plant Physiol **38:** 179–204

Beffa RS, Hofer R-M, Thomas M, Meins F (1996) Decreased susceptibility to viral disease of β-1,3-glucanase-deficient plants generated by antisense transformation. Plant Cell **8:** 1001–1011

Bruce RJ, West CA (1989) Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. Plant Physiol **91:** 889–897

Chabbert B, Monties B, Liu T-T Y, Lagrimini LM (1992) Lignin content and composition in transgenic tobacco plants with altered peroxidase activity. *In* M Kuwahara, M Shimada, eds, Proceedings of the Fifth International Conference for Biotechnology in the Pulp and Paper Industry. Uni Publishers, Tokyo, Japan, pp 481–485

Cohen JD, Bandurski RS (1978) The bound auxins: protection of indole-3-acetic acid from peroxidase-catalyzed oxidation. Planta **139:** 203–208

Dean JFD, Eriksson K-EL (1994) Laccase and the deposition of lignin in vascular plants. Holzforschung **48:** 21–33

Dowd PF, Lagrimini LM (1997) The role of peroxidase in host insect defenses. *In* N Carozzi, M Koziel, eds, Advances in Insect Control: The Role of Transgenic Plants. Taylor and Francis, New York, pp 195–223

Dunford HB (1991) Horseradish peroxidase: structure and kinetic properties. *In* J Everse, KE Everse, MB Grisham, eds, Peroxidases in Chemistry and Biology, Vol II. CRC, Boca Raton, FL, pp 1–24

Epstein E, Cohen JD, Bandurski RS (1980) Concentration and metabolic turnover of indoles in germinating kernels of *Zea mays* L. Plant Physiol **65:** 415–421

Felton GW, Duffey SS (1991) Protective action of midgut catalase in lepidapteran larvae against oxidative plant defenses. J Chem Ecol **17:** 1715–1732

Flachmann R, Kuehlbrandt W (1995) Accumulation of plant antenna complexes is regulated by post-transcriptional mechanisms in tobacco. Plant Cell **7:** 149–160

Fry SC (1986) Cross-linking of matrix polymers in the growing cell walls of angiosperms. Annu Rev Plant Physiol **37:** 165–186

Garner CW (1984) Peroxidation of free and esterified fatty acids by horseradish peroxidase. Lipids **19:** 863–870

Gazaryan IG, Lagrimini LM (1996) Tobacco anionic peroxidase overexpressed in transgenic plants. II. Aerobic oxidation of indole-3-acetic acid. Phytochemistry **42:** 1271–1278

Gazaryan IG, Lagrimini LM, Ashby GA, Thorneley RNF (1996) The mechanism of indole-3-acetic acid oxidation by plant peroxidases. Anaerobic stopped-flow spectrophotometric studies on horseradish and tobacco peroxidases. Biochem J **313:** 841–847

Grambow HJ, Langenbeck-Schwich B (1983) The relationship between oxidase activity, peroxidase activity, hydrogen peroxidase, and phenolic compounds in the degradation of indole-3-acetic acid *in vitro*. Planta **157:** 131–137

Grisebach H (1981) Lignins. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants. Academic Press, New York, pp 457–477

Harkin JM, Obst JR (1973) Lignification in trees: indication of exclusive peroxidase participation. Science **180:** 296–298

Hewelt A, Prinsen E, Schell J, Van Onckelen H, Schmülling T (1994) Promoter tagging with a promoterless *ipt* gene leads to cytokinin-induced phenotypic variability in transgenic tobacco plants: implications of gene dosage effects. Plant J **6:** 879–891

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science **227:** 1229–1231

Jiang CZ, Rodermel SR (1995) Regulation of photosynthesis during leaf development in RbcS antisense DNA mutants of tobacco. Plant Physiol **107:** 215–224

Khandjian EW (1987) Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. Biotechnology **5:** 165–167

Kirk TK, Connors WJ, Bleam RD, Hackett WF, Zeikus JG (1975) Preparation and microbial decomposition of synthetic [^{14}C]lignins. Proc Natl Acad Sci USA **72:** 2515–2519

Klotz KL (1995) Expression and regulation of the tobacco anionic peroxidase gene. Dissertation. The Ohio State University, Columbus

Kuipers AGJ, Soppe WJJ, Jacobsen E, Visser RGF (1995) Factors affecting the inhibition by antisense RNA of granule-bound starch synthase gene expression in potato. Mol Gen Genet **246:** 745–755

Lagrimini LM (1991) Wound-induced deposition of polyphenols in transgenic plants overexpressing peroxidase. Plant Physiol **96:** 577–583

Lagrimini LM, Bradford S, Rothstein S (1990) Peroxidase-induced wilting in transgenic tobacco plants. Plant Cell **2:** 7–18

Lagrimini LM, Burkhart WA, Moyer MB, Rothstein S (1987) Molecular cloning of complementary DNA encoding the ligninforming peroxidase from tobacco: molecular analysis and tissue specific expression. Proc Natl Acad Sci USA **84:** 7542–7546

Lagrimini LM, Joly RJ, Dunlap JR, Liu TY (1997) The consequence of peroxidase overexpression in transgenic plants on root growth and development. Plant Mol Biol **33:** 887–895

Lagrimini LM, Rothstein S (1987) Tissue specificity of tobacco peroxidase isozymes and their induction by wounding and tobacco mosaic virus infection. Plant Physiol **84:** 438–442

Lagrimini LM, Vaughn J, Erb WA, Miller SA (1993) Peroxidase overproduction in transgenic tomato plants: analysis of wound-induced polyphenol deposition and disease resistance. Hortscience **28:** 218–221

Lehrach H, Diamond D, Wozney JM, Boedtker H (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions: a critical reexamination. Biochemistry **16:** 4743–4750

Mäder M, Füssl R (1982) Role of peroxidase in lignification of tobacco cells. Plant Physiol **70:** 1132–1134

Mäder M, Nessel A, Bopp M (1977) On the physiological significance of the isoenzyme groups of peroxidase from tobacco demonstrated by biochemical properties. Z Pflanzenphysiol **82:** 247–260

Picton S, Barton SL, Bouzayen M, Hamilton AJ, Grierson D (1993) Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene. Plant J **3:** 469–481

Powell PA, Stark DM, Sanders PR, Beachy RN (1989) Protection against tobacco mosaic virus in transgenic plants that express tobacco mosaic virus antisense RNA. Proc Natl Acad Sci USA **86:** 6949–6952

Rodermel SR, Abbott MS, Bogorad L (1988) Nuclear-organelle interaction: nuclear antisense gene inhibits ribulose bisphosphate carboxylase enzyme levels in transformed tobacco plants. Cell **55:** 673–681

Romano CP, Hein MB, Klee HJ (1991) Inactivation of auxin in tobacco transformed with the indoleacetic acid-lysine synthetase gene of *Pseudomonas savastanoi*. Genes Dev **5:** 438–446

Rothstein SJ, Lagrimini LM (1989) Silencing gene expression. Oxf Surv Plant Mol Cell Biol **6:** 55–78

Rothstein SJ, Lahners KN, Lotstein RJ, Carozzi NB, Jayne SM, Rice DA (1987) Promoter cassettes, antibiotic-resistance genes, and vectors for plant transformation. Gene **53:** 153–161

Sherf BA, Bajar AM, Kolattukudy PE (1993) Abolition of an inducible highly anionic peroxidase activity in transgenic tomato. Plant Physiol **101:** 201–208

Sitbon F, Hennion S, Sundberg B, Little CHA, Olsson O, Sandberg G (1992) Transgenic tobacco plants coexpressing the *Agrobacterium tumefaciens iaaM* and *iaaH* genes display altered growth and indoleacetic acid metabolism. Plant Physiol **99:** 1062–1069

Smith JA, Hammerschmidt R (1988) Comparative study of acidic peroxidases associated with induced resistance in cucumber, muskmelon and watermelon. Physiol Mol Plant Pathol **33:** 255–261

Tenhaken R, Levine A, Brisson LF, Dixon RA, Lamb C (1995) Function of the oxidative burst in hypersensitive disease resistance. Proc Natl Acad Sci USA **92:** 4158–4163

Teutonico RA, Dudley MW, Or, JD, Lynn DG, Binns AN (1991) Activity and accumulation of cell division-promoting phenolics in tobacco tissue cultures. Plant Physiol **97:** 288–297

Ward ER, Uknes SJ, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl-Goy P, Métraux J-P, Ryals JA (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3:** 1085–1094

Welinder KG (1992) Superfamily of plant, fungal and bacterial peroxidases. Curr Opin Struc Biol **2:** 388–393

Whitmore FW (1978) Lignin-carbohydrate complex formed in isolated cell walls of callus. Phytochemistry **17:** 421–425