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Peroxidase-Induced Wilting in Transgenic Tobacco Plants

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Peroxidases are a family of isoenzymes found in all higher plants. However, little is known concerning their role in growth, development, or response to stress. Plant peroxidases are heme-containing monomeric glycoproteins that utilize either H_2O_2 or O_2 to oxidize a wide variety of molecules. To obtain more information on possible in planta functions of peroxidases, we have used a cDNA clone for the primary isoenzyme form of peroxidase to synthesize high levels of this enzyme in transgenic plants. We were able to obtain *Nicotiana tabacum* and *N. sylvestris* transformed plants with peroxidase activity that is 10-fold higher than in wild-type plants by introducing a chimeric gene composed of the cauliflower mosaic virus 35S promoter and the tobacco anionic peroxidase cDNA. The elevated peroxidase activity was a result of increased levels of two anionic peroxidases in *N. tabacum*, which apparently differ in post-translational modification. Transformed plants of both species have the unique phenotype of chronic severe wilting through loss of turgor in leaves, which was initiated at the time of flowering. The peroxidase-induced wilting was shown not to be an effect of diminished water uptake through the roots, decreased conductance of water through the xylem, or increased water loss through the leaf surface or stomata. Possible explanations for the loss of turgor, and the significance of these types of experiments in studying isoenzyme families, are discussed.

INTRODUCTION

The development of techniques to alter the expression of identified plant genes through transformation is potentially an important tool for the analysis of the physiological role of the gene product. This is especially important if it has proven difficult to delineate enzyme function using standard biochemical and molecular techniques. Presented here is an example of an enzyme—peroxidase—that has been identified for some time. The gene has recently been characterized, but many of the natural products and reactants remain to be determined. It should be possible to learn a great deal about in vivo enzyme reactions by either increasing or decreasing the expression of an isolated gene and analyzing any resulting alteration in phenotype. In this paper, a gene coding for the tobacco anionic peroxidase isoenzyme was overexpressed in tobacco plants with unpredicted effects on the phenotype of the transformed plants.

Plant peroxidases (EC 1.11.1.7, donor:hydrogen-peroxide oxidoreductase, POD) are among the most studied but least understood of all plant proteins (Gaspar et al., 1982). Their universal nature and the availability of many sensitive colorimetric assays have made peroxidase a convenient enzyme marker in genetic, physiological, and pathological studies (Greppin, Penel, and Gaspar, 1986; van Huystee, 1987). Since its identification near the turn of the century, peroxidase has been implicated in polysaccharide cross-linking (Fry, 1986), indole-3-acetic acid oxidation (Hinnman and Lang, 1965), cross-linking of extensin monomers (Everdeen et al., 1988), lignification (Grisebach, 1981), wound-healing (Espelie, Franceschi, and Kolattukudy, 1986), phenol oxidation (Schmid and Feucht, 1980), pathogen defense (Hammerschmidt, Nuckles, and Kuc, 1982), and the regulation of cell elongation (Goldberg et al., 1986). Although peroxidase is a highly catalytic enzyme, it has very little specificity, and there exists a multitude of isoenzyme forms (Gaspar et al., 1982). This makes it difficult to understand the actual in planta functions of peroxidase and the role this enzyme plays in plant growth and adaptation to its environment.

The molecular analysis of peroxidases began with the cloning and characterization of the anionic peroxidase isoenzyme from tobacco (Lagrimini et al., 1987). The tobacco anionic peroxidase (M_r 36,000, pI 3.5) is the best characterized of all tobacco peroxidases (Mäder, Nessel, and Bopp, 1977; Nessel and Mäder, 1977; Mäder, Ungemach, and Schloss, 1980; Lagrimini and Rothstein, 1987).

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The anionic peroxidase accounts for nearly 90% of peroxidase activity in all tissues with the exception of root, and this isoenzyme was found in all developmental stages of the plant (Lagrimini and Rothstein, 1987). The anionic isoenzyme is extracellular and can be found in xylem, sclerenchyma, and epidermal tissue. It has been shown to catalyze efficiently the polymerization of cinnamyl alcohols into lignin in vitro (Mäder et al., 1980; Mäder and Fussl, 1982). These and other observations have led to the conclusion that this isoenzyme is necessary for the formation of lignin. The functions of this isoenzyme in epidermal tissue and details of the developmental expression of the isoenzyme remain to be determined.

Any analysis of peroxidase function must take into account that peroxidase is a highly catalytic enzyme with a large number of potential substrates in the plant. Many of the reaction products are toxic, and the activity of the enzyme needs to be closely controlled. Much of this control is not at the level of substrate specificity but, rather, at the level of gene expression, cellular localization, and the availability of substrates. Understanding how individual peroxidase isoenzymes function in the plant will be achieved by determining natural reactants and products and localizing isoenzymes and reactants during the development of plants that have suppressed isoenzyme levels through mutation or antisense RNA. To better understand how the tobacco anionic peroxidase may function in vivo, we have altered the level of a single peroxidase isoenzyme in transgenic plants. We describe how peroxidase expression was increased 10-fold by introducing a chimeric anionic peroxidase gene into tobacco via *Agrobacterium*-mediated transformation. Phenotypes resulting from peroxidase overproduction and in vivo enzymatic function will also be discussed.

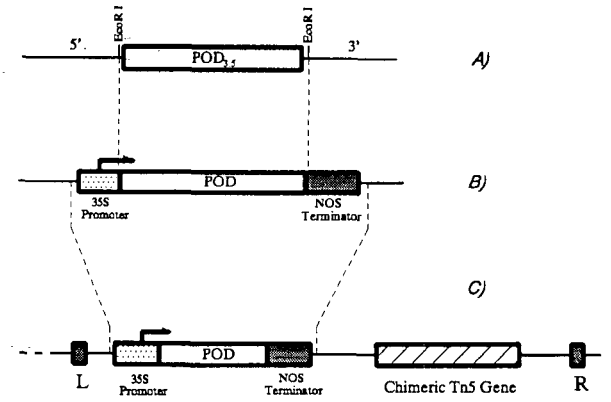

Figure 1. Construction of pML507, the Tobacco Anionic Peroxidase Overexpression Vector.

(A) The 1256-bp tobacco anionic peroxidase cDNA insert was isolated by EcoRI restriction endonuclease digestion of the plasmid $pPOD_{3.5}$ (Lagrimini et al., 1987). The 3′ recessed ends were filled in with Klenow DNA polymerase, and BamHI linkers were added with T_4 DNA ligase.

(B) This fragment was subsequently cloned into the BamHI site of pCib710 between the CaMV 35S transcription promoter and terminator (Rothstein et al., 1987b). A recombinant plasmid that possesses the anionic peroxidase coding strand in the correct orientation with respect to the CaMV 35S promoter was selected by restriction enzyme analysis.

(C) The chimeric 35S::$POD_{3.5}$ gene was excised with SalI and SacI and inserted into pCib10. pCib10 contains the neomycin phosphotransferase gene of Tn5 (*NPTII*) fused to the nopaline synthase promoter and terminator sequences and the T-DNA left and right borders (Rothstein et al., 1987b). This provides kanamycin resistance to transformed plants. The resulting plasmid was called pML507.

RESULTS

Vector Construction and the Preparation of Transgenic Tobacco Plants

The 1256-bp tobacco anionic peroxidase cDNA insert was removed from the plasmid $pPOD_{3.5}$ by restriction endonuclease digestion with EcoRI (Lagrimini et al., 1987). This fragment contains the entire anionic peroxidase coding sequence, including the 22-amino acid signal peptide facilitating secretion into the cell wall space and the poly(A) addition site. After the addition of BamHI linkers, the peroxidase cDNA was inserted into the BamHI site of pCib710 containing the cauliflower mosaic virus (CaMV) 35S promoter/terminator cassette, as shown in Figure 1. This construct should direct the high-level expression of the tobacco anionic peroxidase in transgenic plants. The 35S::$POD_{3.5}$ construct was excised with SacI and SalI, and then inserted into the binary vector plasmid pCib10 (Rothstein et al., 1987). This plasmid permits replication in *Agrobacterium tumefaciens* and will facilitate the transfer of the peroxidase chimeric gene into tobacco cells. This plasmid also confers kanamycin resistance in plants, which permits the selection of transformed plants. The resulting peroxidase overproducer plasmid, pPOD507, was introduced into the *A. tumefaciens* strain A136 containing the Ti helper plasmid pCib542 and used to transform leaf discs from *Nicotiana sylvestris* and *N. tabacum* cv Coker 176 via co-cultivation (described in Methods). Transgenic tobacco plants were obtained by selection on kanamycin in culture. After 7 weeks, 6 to 12 kanamycin-resistant plants were potted in soil-less media and transferred to the greenhouse where they were propagated further.

Assay of Peroxidase Levels and Isoenzyme Distribution in Transgenic Plants

Leaf tissue from 20-cm-tall kanamycin-resistant plants was assayed for total peroxidase activity. Table 1 shows that

Table 1. Peroxidase Activity from Randomly Selected Transgenic Plants

	N. sylvestris	*N. tabacum*
Wild type	59.5	61.8
	60.0	68.3
507	676.0	667.0
	309.0	445.0
	672.0	609.0
	118.0*	201.0

Leaf tissue from 20-cm-tall regenerated *N. sylvestris* and *N. tabacum* plants that were selected on kanamycin was assayed for peroxidase activity. Peroxidase activity is expressed in guaiacol units per gram, fresh weight. Total protein concentration was found not to vary by more than 10%. Plants marked wild type were transformed with pCib10, which does not include the chimeric peroxidase gene. Four plants that were transformed with POD507 were selected from each species. All plants demonstrated the wilting phenotype with the exception of the individual marked (*).

peroxidase activity from both transgenic *N. tabacum* and *N. sylvestris* plants was twofold to 10-fold higher than that seen in wild-type plants. Isoelectric focusing was performed to confirm that the elevated peroxidase activity was the result of increased synthesis of the anionic isoenzymes, as would be predicted. Isoelectric focusing polyacrylamide gel electrophoresis of a leaf extract from *N. tabacum* plant 507C shows that both anionic isoenzymes were found at elevated levels as compared with other peroxidase isoenzymes, as illustrated in Figure 2. This result indicated that the elevated peroxidase activity was a consequence of the introduced peroxidase chimeric gene. Isoelectric focusing analysis also shows that both anionic peroxidase isoenzymes of *N. tabacum* come from a single gene and differ in isoelectric point as a result of some type of post-translational modification.

To obtain plants for further analysis, several transgenic *N. tabacum* and *N. sylvestris* plants with 10-fold elevated peroxidase activity were self-pollinated, and seeds were collected. These seeds were sowed on agar, and leaf tissue was assayed for peroxidase activity when the seedlings were 5 mm tall. Approximately 25% of the seedlings had wild-type peroxidase activity and died when transferred to agar with kanamycin. Elevated peroxidase activity paralleled kanamycin resistance, providing further evidence that elevated peroxidase activity was a consequence of the chimeric POD507 gene. Segregation of peroxidase overexpression and kanamycin resistance appeared to occur in a Mendelian fashion. However, it was difficult to distinguish those plants that were homozygous for the chimeric gene (POD507/POD507) from those that were single copy (POD507/WT). This was determined by self-fertilizing the F1 plants that were kanamycin-resistant and plating the F2 generation seeds on agar plus kanamycin. Seeds resulting from those plants that were kan^R/kan^R, and presumably POD507/POD507, exhibited 100% survival on kanamycin. Seeds from kan^R/WT plants resulted in 75% survival. A single transgenic plant from each species of tobacco, *N. sylvestris* 507J and *N. tabacum* 507C, homozygous for the chimeric peroxidase gene, was selected for further studies. Both of these transgenic plants have 10-fold higher peroxidase activity than wild-type tobacco. Peroxidase activity was found at 10-fold over wild-type in leaf tissue from the youngest seedling to maturity.

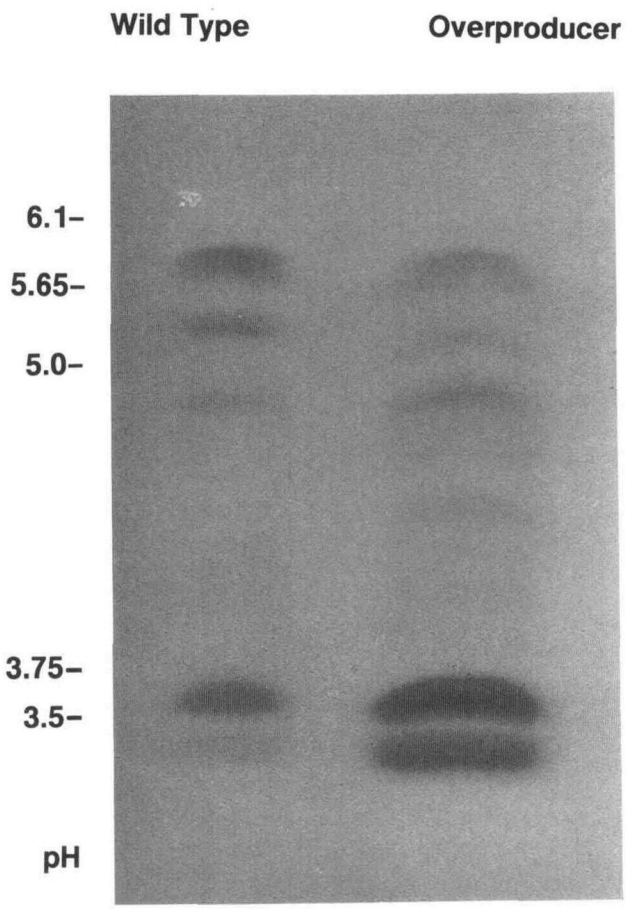

Figure 2. Peroxidase Isoenzymes from a Selected Peroxidase Overproducer Transgenic Plant.

Leaf tissue from the offspring of a single self-fertilized transgenic *N. tabacum* plant (507C) was analyzed for peroxidase isoenzymes. Extract from 0.1 g of leaf tissue of wild-type or peroxidase overproducer plants (507C) was subjected to isoelectric focusing, followed by staining for peroxidase isoenzymes. Isoelectric points for peroxidase isoenzymes are marked to the left of the gel.

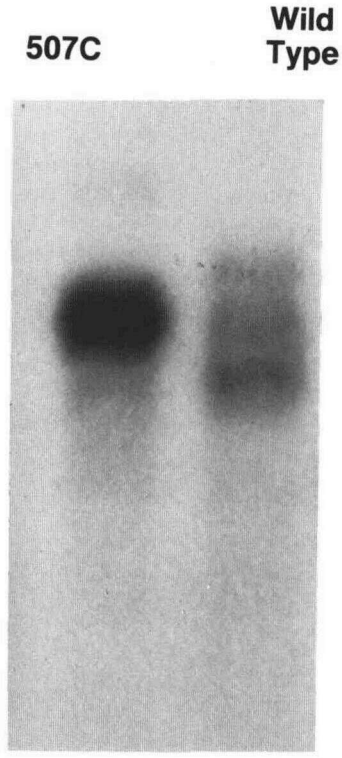

Figure 3. Messenger RNA Levels from a Wild-Type and a Transformed Plant.

Leaf poly(A)$^+$ RNA (1 μg) from an *N. tabacum* wild-type or transgenic plant 507C, overexpressing the anionic peroxidase by 10-fold over wild type, was blotted onto nylon filters and probed with the anionic peroxidase cDNA clone. Filters were subjected to autoradiography for 48 hr at −80°C with an intensifying screen (DuPont Cronex).

Messenger RNA Levels in Transgenic Plants

The steady-state accumulation of anionic peroxidase messenger RNA in wild-type and *N. tabacum* 507C plants was analyzed by RNA gel blot analysis. Poly(A)$^+$ RNA was isolated from young leaf tissue of both wild-type and 507C plants, subjected to agarose-formaldehyde gel electrophoresis, and then transferred to a nylon filter. The filter was hybridized with the anionic peroxidase cDNA clone $pPOD_{3.5}$. A typical blot from such an experiment is shown in Figure 3. The anionic peroxidase mRNA was found at extremely low levels in leaf tissue, as previously reported by Lagrimini et al. (1987). The RNA was heterogeneous, as can be seen by the presence of two to three bands. In contrast, the mRNA isolated from *N. tabacum* 507C leaf tissue shows a single intense band representing the chimeric anionic peroxidase mRNA. Estimates by densitometric scanning reveal the signal in the transformed plant to be eightfold to 10-fold higher than that of the wild-type. This demonstrates a strong correlation between anionic peroxidase mRNA levels and isoenzyme activity in transgenic plants.

Altered Phenotype as a Result of the Overproduction of Peroxidase

Those transformed plants with greater than a twofold increase in peroxidase expression displayed a very distinctive phenotype. They resembled wild-type plants until the onset of flowering, at which point they began wilting. In this context, the onset of flowering is defined as the visible appearance of flower buds. This was seen in several *N. tabacum* and *N. sylvestris* transformed plants with high levels of peroxidase (>twofold) and could not be alleviated by increased frequency of watering. In all cases, the wilting phenotype co-segregated with both elevated peroxidase activity and kanamycin resistance. The severity of wilting showed some correlation with the level of peroxidase overproduction. The wilting phenomenon was not seen in wild-type, mock-transformed, or transgenic plants with less than twofold elevated peroxidase activity. Figure 4 shows a series of photographs of a wild-type and an *N. sylvestris* 507J plant. Three days prior to the onset of wilting, both plants appear very similar; however, the leaves and internodes in the 507J were slightly smaller. Three days after the onset of wilting, the wilting phenotype can be seen clearly in the 507J plant. At this point, the 507J plant will recover completely during the night, and will only begin wilting after sunrise. The final photograph in Figure 4 was taken 4 weeks following the onset of wilting and shows the dramatic effect of chronic wilting. The growth of 507J was severely stunted, presumably as a consequence of the wilting. Also, in the later stages of wilting, the leaves would not regain turgor during the night. Stunting was proportionally less severe in plants with less than 10-fold overproduction of peroxidase.

The two species *N. tabacum* and *N. sylvestris* differ physiologically in that *N. tabacum* will flower only after the completion of vertical growth, whereas *N. sylvestris* progresses through most of its vertical growth concurrent with flowering. This developmental difference contributes to the observation that *N. tabacum* 507C reaches normal size before beginning to wilt. A mature wild-type *N. tabacum* plant is compared with a 507C plant in Figure 5. Because wilting began only after the completion of vertical growth, the transformed *N. tabacum* plants achieved normal height.

Measurements of Water Loss and Stomatal Aperture

Peroxidase-induced wilting could be explained by a decrease in water uptake or transport or an increased loss

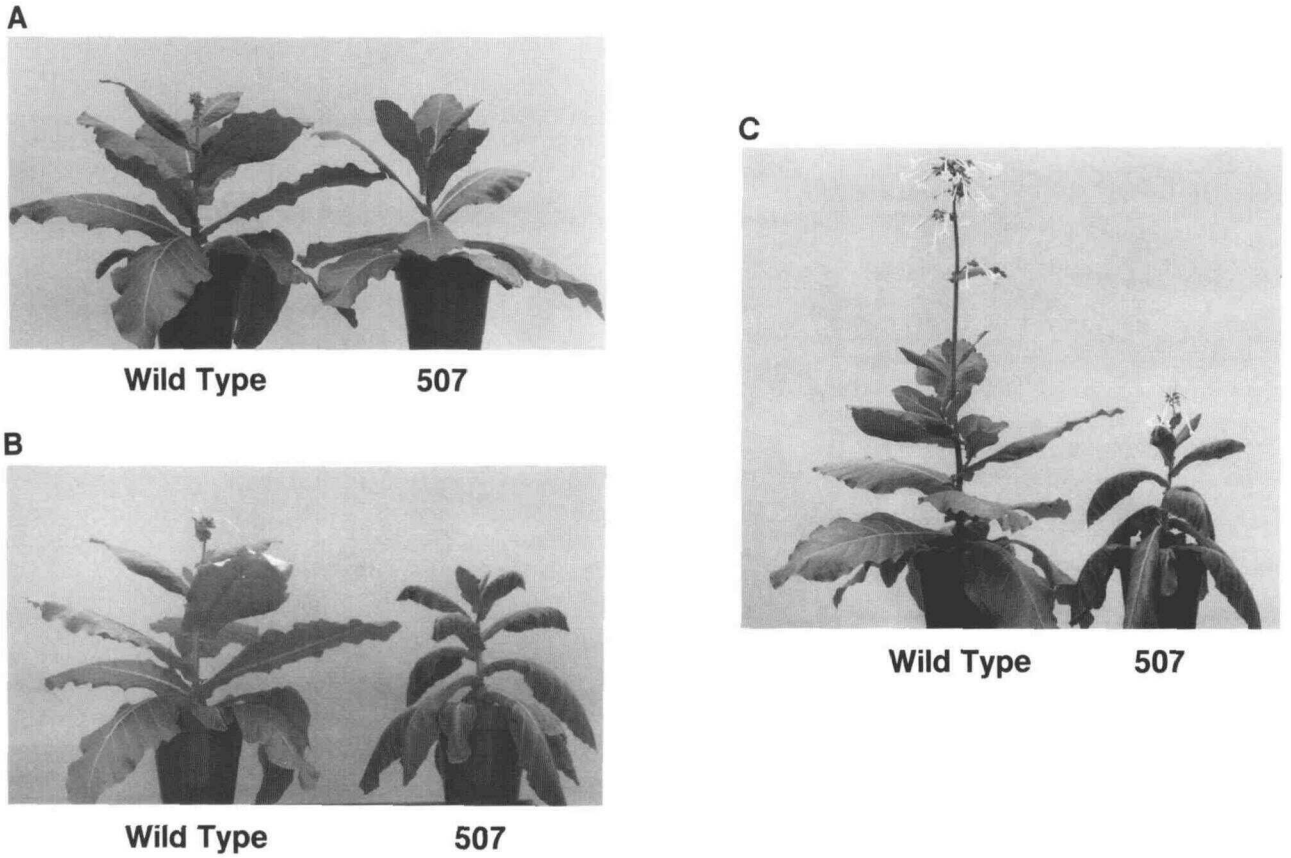

Figure 4. *N. sylvestris* Wild-Type and Peroxidase Overproducer 507J Plants.

The same two greenhouse-grown plants were photographed as follows:
(A) Three days preceding the onset of wilting.
(B) Three days after the onset of wilting.
(C) Four weeks following the onset of wilting.

of water through the leaf surface or through the stomata, which exceeds water uptake. To test for the former, *N. tabacum* 507C plants were placed horizontally to promote the formation of axillary shoots. Leaves from the lateral shoots had normal turgor even though the primary shoot was wilted (data not shown). The secondary shoots only lost turgor when flower buds were initiated on them. Also, detached leaves from wilting 507C plants placed in water remained wilted when in direct sunlight. The detached leaves recovered turgor when placed in subdued light. To test for water loss through the stomata, the leaves of mature wild-type *N. tabacum* plants and *N. tabacum* 507C plants were monitored for stomatal conductance and transpiration rate with a steady-state leaf porometer. This device directly measures water loss through the leaf surface. These measurements are presented in Table 2. The mean stomatal conductance in control leaves was 0.758 sec cm^{-1}, and the mean transpiration rate was 12.36 mg H_2O m^{-2} sec^{-1} at mid-day in direct sunlight. Stomatal conductance and transpiration rate were measured at the same time in *N. tabacum* 507C plants that were wilted. The mean stomatal conductance was reduced eightfold at 0.10 sec cm^{-1}, and the mean transpiration rate was 1.54 mg H_2O m^{-2} sec^{-1}. Before the establishment of the wilting phenotype, stomatal conductance was similar to that of a wild-type plant (0.802 sec cm^{-1}). This eightfold reduction in stomatal conductance indicated that there was not only no increase in water loss, but there was actually a decrease in water loss through the leaf surface.

To determine whether the stomata were open or closed in the wilted leaf, the stomata were directly examined under the microscope. The lower epidermis from wild-type or wilted plants was quickly removed and immediately placed in immersion oil on a glass slide to arrest the

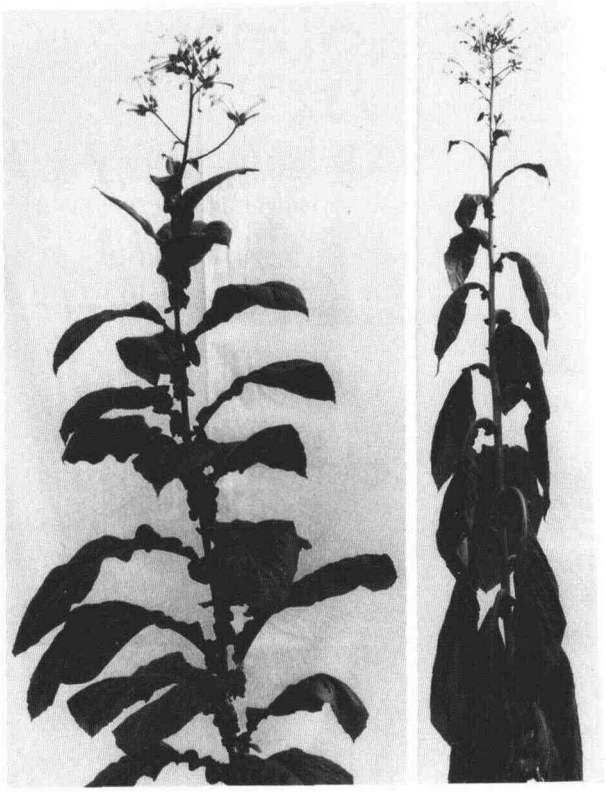

Figure 5. *N. tabacum* Wild-Type Compared with the 507C Transformed Plant.

These plants were photographed 1 week following the onset of wilting.

stomata in their current position (Fisher, 1968). The stomata were examined under a phase-contrast microscope, and, as seen in Figure 6, the stomata in the transformed plant were closed compared with the wild-type plant. These two observations confirm that the peroxidase-induced wilting was not a result of water loss through the leaf surface.

Histological Examination of Xylem

To determine whether the wilting phenotype is a direct result of abnormal xylem morphology, stem sections were subjected to microscopic analysis. Several hundred cross-sections through stem tissue from wild-type or wilted *N. sylvestris* transformed plants revealed similar vessel number and structure. Typical sections are shown in Figure 7. The same similarity was also observed in vessel longitudinal examination, with similar ratios of helical and pitted vessels in wild-type and peroxidase overproducer plants (Figure 7).

DISCUSSION

The ability to generate transgenic plants has provided a powerful tool to increase our understanding of physiological processes. Peroxidases have been difficult to study through classical genetics and biochemistry. There are a large number of peroxidase isoenzymes and even more peroxidase genes. Peroxidases are a convenient physiological marker because of the simplicity of their activity assay, and this feature has allowed peroxidase to be a useful marker of plant development, physiology, infection, and stress (Thorpe, Van, and Gaspar, 1978; Greppin et al., 1986). The available molecular and biochemical details of peroxidase function do not provide a clear picture of how the different peroxidase isoenzymes function in plant development and the stress response. We are utilizing transgenic plants that have had both the steady-state levels and tissue distribution of the anionic peroxidase altered to assist in the thorough characterization of peroxidase function in the plant. The anionic peroxidase of tobacco, which at the very least is involved in lignin synthesis, has had its protein-coding sequence inserted into the tobacco genome under the control of the CaMV 35S promoter. This promoter has been shown to be active in phloem, epidermis, and, to a lesser extent, in other tissues (Jefferson, Kavanagh, and Bevan, 1987). This compares with the preferential expression of the native anionic peroxidase gene in immature xylem and epidermis (S. Bradford and L.M. Lagrimini, unpublished results).

Transgenic tobacco plants were shown to have an increase in total peroxidase activity that was accounted for by the specific overproduction of the anionic isoenzymes. Different transformed plants had peroxidase activity increased by as little as twofold to greater than 10-fold. This variability is common in *Agrobacterium* transformed plants

Table 2. Measurements of Leaf Stomatal Conductance and Transpiration Rate of Transformed Plants

	Stomatal Conductance (sec cm^{-2})	Transpiration Rate (mg H_2O $m^{-2}sec^{-1}$)
Wild type	0.758 ± 0.08	12.36 ± 1.54
507C before wilting	0.802 ± 0.1	13.45 ± 1.23
507C after wilting	0.1 ± 0.03	1.54 ± 0.37

Values are presented as the average from five leaves measured in mid-afternoon direct sunlight. Peroxidase overproducer plant *N. tabacum* 507C was monitored 1 week prior to wilting and 1 week after the onset of wilting.

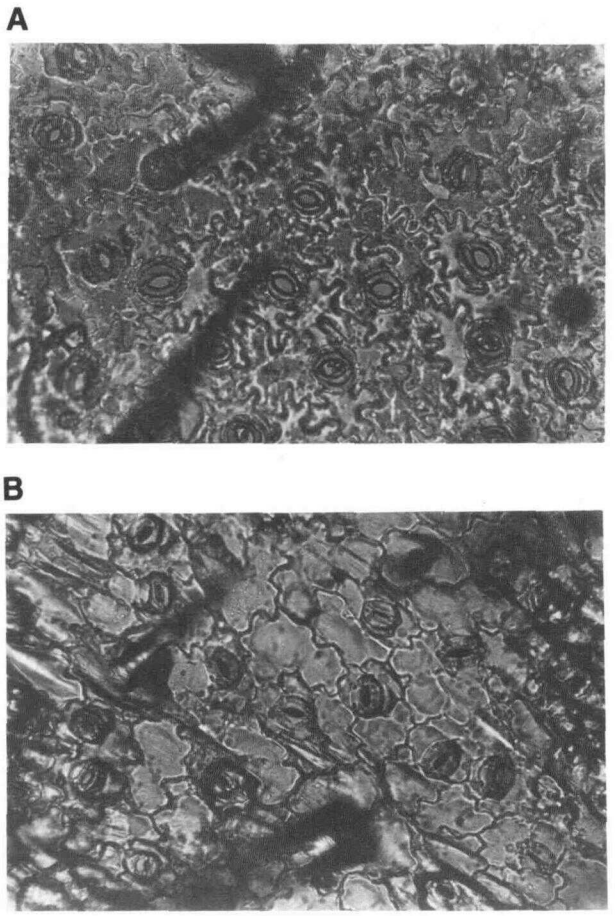

Figure 6. Stomata from Either Wild-Type or Transformed Tobacco Plants.

The lower epidermis was peeled away from fully expanded leaves. The tissue was placed on microscope slides in immersion oil and photographed under phase contract microscopy.
(A) Wild-type *N. tabacum* plant.
(B) 507C transformed *N. tabacum* plant.

and is attributed to gene copy number and position effects (Rothstein et al., 1987a; van der Krol et al., 1988). Analysis of mRNA levels in control and transformed plants demonstrated elevated anionic peroxidase mRNA levels that correspond to the increased enzymatic activity. Messenger RNA from wild-type *N. tabacum* leaf tissue is composed of a heterogeneous population of RNAs that hybridizes to the anionic peroxidase cDNA clone. There were three RNAs detected with sizes of 1000, 1200, and 1400 nucleotides. The signal was very weak in leaf tissue. In pith tissue, a single strong hybridization was detected at 1200 nucleotides (Lagrimini et al., 1987). We have not determined the source of the three hybridizations in leaf tissue; however, it is likely a consequence of heterogeneity among the four copies of the anionic peroxidase gene. As predicted, the 507C plant possessing the chimeric POD507 gene shows a strong hybridization at 1200 nucleotides. We cannot explain at this time why the other two weak hybridizations are not seen in the transformed plants.

Part of the difficulty in studying peroxidases is the large number of isoenzymes. As many as 35 isoenzymes have been detected in higher plants. The number could directly represent gene copy number through polymorphisms, post-translational polymorphisms, or artifacts of preparation such as cross-linking via polyphenols. The allotetraploid species *N. tabacum* was the result of an interspecific hybridization between *N. sylvestris* and *N. tomentosiformis* approximately 1 million years ago (Okamuro and Goldberg, 1985). *N. tabacum* retains both pairs of parental chromosomes, causing peroxidase isoenzyme patterns and anionic peroxidase gene patterns to be the sum of the two parental patterns (Lagrimini et al., 1987). *N. tabacum* has two anionic peroxidase isoenzymes, $POD_{3.5}$ (M_r 35,000) and $POD_{3.75}$ (M_r 37,000), in approximately equal amounts (Lagrimini and Rothstein, 1987). One or the other isoenzyme is predominant in each parent species (>95%) (S. Bradford and L.M. Lagrimini, unpublished results). *N. tabacum* has four copies of the anionic peroxidase gene, two from each parent. Both species, *N. tabacum* and *N. sylvestris*, were transformed with pPOD507 to confirm suspicions that the two isoenzymes were the result of post-translational polymorphisms. This was confirmed because the overproduction of a single peroxidase chimeric gene in transgenic *N. tabacum* plants resulted in the increase of both anionic isoenzymes (Figure 2) and the increase of a single isoenzyme in *N. sylvestris* (data not shown). Apparently, both parental species have enzymes that preferentially modify the anionic peroxidase peptide, whereas *N. tabacum* has both enzyme systems. This confirms that at least a portion of the complexity of peroxidase isoenzyme profiles was a result of protein modification, as opposed to gene number. It is hoped that, with further molecular studies, a complex family of >12 isoenzymes in *N. tabacum* will be simplified to three or four functionally distinct isoenzyme groups.

The first noticeable difference in transformed plants overproducing peroxidase was the development of chronic wilting that coincided with flower bud initiation. We conclude that wilting is a direct result of the overproduction of peroxidase in transformed plants on the basis of the following observations: Wilting occured only in those plants transformed with POD507 and not in those plants transformed with vector only. Also, wilting was seen only in those transformed plants with greater than twofold elevated peroxidase activity, and the wilting phenotype was seen in several transformants in two species of tobacco, *N. tabacum* and *N. sylvestris*. Plants initially appeared completely normal except that the leaves of the transgenic plants were slightly smaller and the plants had slightly

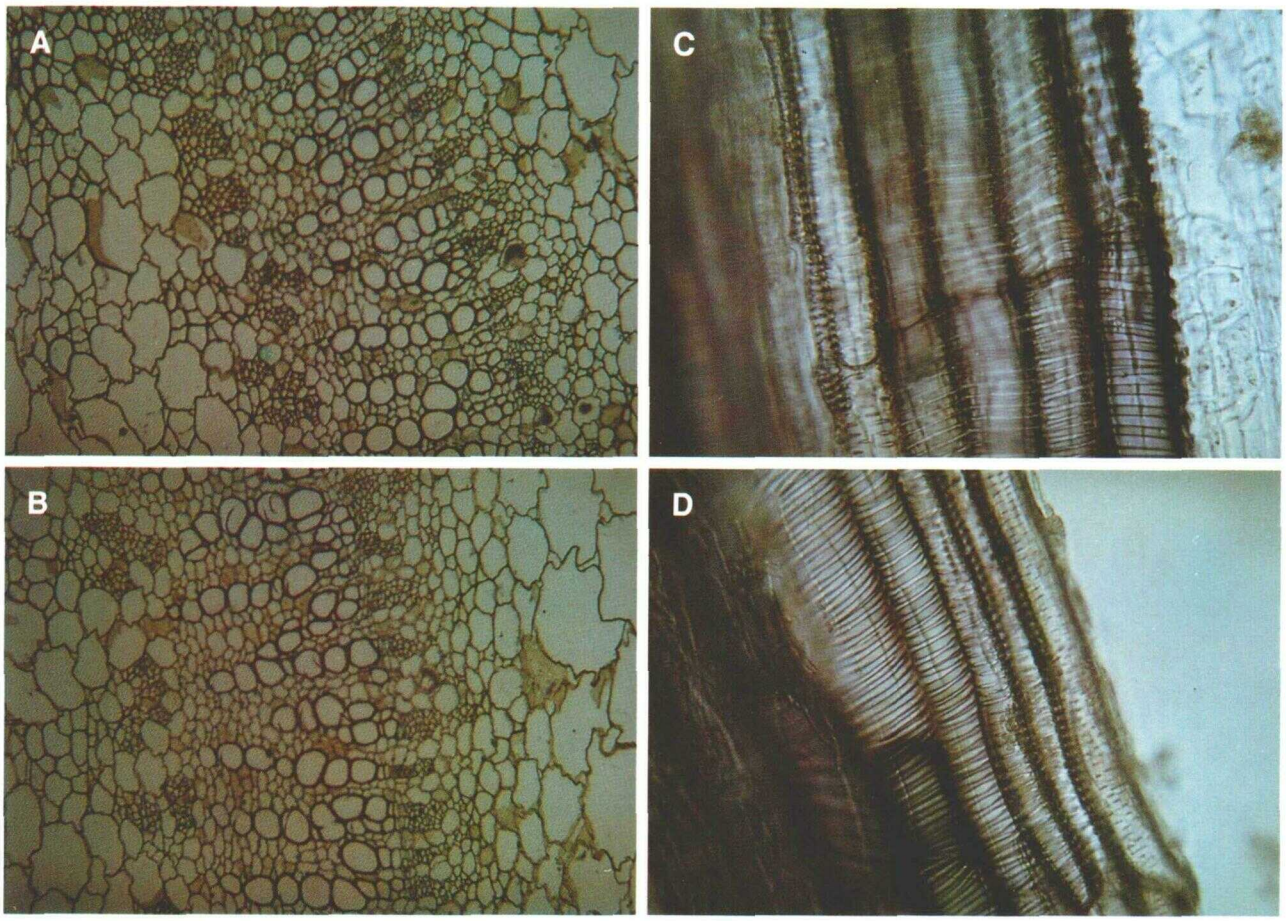

Figure 7. Light Microscopic Histological Examination of Stem Tissue.

(A) 13-μm cross-section of wild-type *N. sylvestris* plant stem tissue.
(B) 13-μm cross-section of wilted *N. sylvestris* 507J plant stem tissue.
Xylem vessels were excised by hand from fresh tissue for longitudinal examination.
(C) *N. sylvestris* wild-type plant xylem tissue.
(D) *N. sylvestris* 507J plant xylem tissue.

shorter internodes. *N. tabacum* differs in its physiology in that the plants grow to their final height before flowering, whereas *N. sylvestris* begins flowering concurrent with the initiation of vertical growth. This physiological difference results in a dramatic reduction in the final plant height in transformed *N. sylvestris*, presumably as a result of the extreme stress placed on the plant due to wilting during the vertical growth phase. Cell elongation is dependent upon turgor pressure to expand cell walls (Boyer, 1985). On the other hand, transformed *N. tabacum* plants approach near normal height before the initiation of flower buds and peroxidase-induced wilting.

During the first 10 days following the onset of wilting, the plants displayed the following physiological characteristics: (1) Peroxidase-induced wilting affected all leaves equally with no indication that wilting began first in either older or younger leaves; (2) the plants wilted only when illuminated under direct sunlight, recovered turgor pressure completely during the night, and wilted only partially on overcast days; and (3) detached leaves placed in water wilted in direct sunlight and recovered when placed in subdued light. Recovery time was 20 min to 30 min, and this cycle could be repeated many times (data not shown). More than 10 days after the onset of wilting, the symptoms became increasingly severe. The leaves lost their ability to recover at night or in subdued light. The plants survived through maturity and produced viable seeds with normal seed set (about 1000 seeds/capsule); however, the total number of flowers was reduced because of a complete absence of axillary inflorescences. Surprisingly, chronic

wilting did not hasten leaf senescence. The leaves remained green, but photosynthesis proceeded at a greatly reduced rate until natural senescence occured (data not shown).

The peroxidase-induced wilting phenotype can be explained by any number of biochemical or biophysical aberrations, including uncontrolled water loss through the leaf surface, the inability to take up water into the leaf cells, or a diminished ability to deliver water to the leaf cells. An example of a well-characterized wilty plant is the *flacca* mutation in *Lycopersicon esculentum* cv Rhinelands ruhm (Tal, 1966). The source of the wilting is a high rate of transpiration caused by stomata that never closed. Abscisic acid, a phytohormone required for the closing of stomata, was later found to be only 17% of the level seen in the wild-type (Tal and Nevo, 1973). Measurements were made on wild-type and wilty tobacco plants through the use of a steady-state leaf porometer that measures stomatal conductance and transpiration rate. Our data indicated that water loss through the wilted leaf was not at an elevated rate, but was eightfold less than wild-type. To confirm that the stomata were indeed closed in the wilted leaf, they were observed directly under the microscope. The stomata of wild-type plants were open at midday, as predicted; however, the stomata of wilted plants were closed. Apparently, the plant was responding normally to the loss of water potential by closing its stomata to curtail any additional water loss.

Individual cells maintain turgor by creating an osmotic potential through the concentration of solutes, predominantly sucrose and salts, within the plasmalemma (Boyer, 1985). If dissolved solutes were not formed because of decreased respiration or were removed rapidly from the solute pool through polymerization, the cell would lose its turgor. There is an example of a single gene recessive osmotic mutation in *Arabidopsis thaliana* that results in wilting (Langridge, 1958). Mutant plants are small and have distorted leaves but can be restored to normal phenotype by growth on high-sucrose media. These plants are found to have unusually low osmotic pressure (Langridge, 1958). Preliminary experiments measuring the osmotic pressure of leaf cell sap in peroxidase overproducing tobacco plants showed normal to slightly elevated osmotic pressure, indicating that wilting may not be due to reduced solutes (data not shown). Low turgor can also be caused by decreased permeability of cells. Experiments are in progress to quantify the permeability of leaf cells.

Loss of turgor can result from decreased uptake by the roots or the impedance of water transport through either the vascular tissue or the apoplast. A wilted mutant of maize has poorly developed or missing vascular bundles (Postlethwait and Nelson, 1957). A wilty dwarf tomato plant is found to have anomalous vessel elements. The end walls between vessel elements are lignified, resulting in reticulated or scalariform perforation plates that produce a resistance to water flow (Alldridge, 1964). We were able to exclude as the cause of wilting both diminished water uptake through the roots or impeded water transport through the shoot of wilted tobacco plants because of the following observations: (1) Detached leaves placed in water wilted in direct sunlight, as did attached leaves. (2) The axillary shoots generated from transformed plants placed horizontally did not wilt even though the primary shoot wilted (data not shown). The roots and primary shoot conducted enough water to maintain normal turgor in the axillary shoots. The axillary shoots only began wilting at the point at which flower buds were initiated. (3) Stem tissue from both wild-type and *N. sylvestris* 507J plants was sampled after the onset of wilting in plants of similar size and age. An exhaustive examination of several hundred sections did not reveal significant differences in tissue morphology. However, if peroxidase overexpression were to have a profound effect on xylem vessel structure, the histological examination of stem tissue of transformed plants would reveal differences. These results demonstrate that water uptake at the roots and transport of water through the primary shoot are not dramatically affected in the transformed plant. It is still very possible that either the release of water from the xylem or the movement of water through the apoplast and between cells within the leaf is affected through the overproduction of peroxidase. We are pursuing further morphological and physiological analysis of leaf tissue from transformed plants with emphasis on the xylem and perforation plates, cell size and shape, and cell wall structure and permeability.

The relationship between the initiation of flower buds and wilting was and is of great interest to us. The following evidence leads us to believe that peroxidase-induced wilting is triggered by flowering: (1) Wilting initiates with the appearance of flower buds in two tobacco species with differing flower physiology, and (2) the signal that triggers wilting originates at the apex of the plant with all leaves wilting simultaneously. The observation that axillary shoots retain turgor even though the primary shoot has wilted supports this contention. The connection between flowering and peroxidase-induced wilting may lie in the availability of peroxidase substrates. The presence of a 10-fold excess of peroxidase in and of itself will not alter cell wall composition. The enzyme requires two substrates to synthesize lignin: cinnamyl alcohol and H_2O_2. The appearance of one or both of these substrates, concurrent with flowering, could be determined by quantifying the levels of free cinnamyl alcohol and H_2O_2 in leaf tissue throughout the life span of a wild-type tobacco plant. Alternatively, other potential substrates for peroxidase other than cinnamyl alcohols could appear in response to flowering. Wilting may not even require the sudden appearance of substrates at flowering. Peroxidase-induced changes may be subtle and revealed only when flowers become the primary sink for nutrients and water (Bernier, Kinet, and Sachs, 1981).

Through the creation of transgenic plants that overproduce a single native enzyme—peroxidase—in many tissues

that typically do not synthesize peroxidase, we have opened the door on several interesting relationships between peroxidase regulation and function. Although peroxidase is abundant and ubiquitous among higher plants, the level of expression and tissue-specific regulation of peroxidase remains critical. These experiments demonstrate that altered peroxidase expression has a dramatic effect on the physiology and morphology of the plant. The appearance of phenotypes that were unpredicted through previous biochemical and physiological studies of peroxidase highlights the tremendous potential of this type of analysis. By altering the expression of enzymes in metabolic pathways, it should be possible not only to delineate which enzyme is rate-limiting, but also to determine whether these enzymes play important roles in other physiological processes.

METHODS

Agrobacterium-Mediated Transformations

The anionic peroxidase overproducer plasmid pML507 was introduced into the *Escherichia coli* mobilizing strain S17-1 by DNA transformation (Simon, Priefer, and Puhler, 1983). The plasmid pML507 was then transferred into *Agrobacterium tumefaciens* strain A136 by conjugation (Rothstein et al., 1987b). *A. tumefaciens* strain A136 contains the disarmed helper Ti plasmid pCib542. Exconjugants were selected by their growth on kanamycin. Plants for transformation experiments (*Nicotiana sylvestris* and *N. tabacum*) were grown from seed in the greenhouse with 14-hr daily light periods. Young, fully expanded leaves were removed from 8-week-old plants and surface-sterilized for 10 min with 10% Chlorox, followed by 70% ethanol. Leaf disc co-cultivations and shoot regenerations were performed as described by Horsch et al. (1985) and Rothstein et al. (1987b). Putative transformants were selected after 3 weeks to 5 weeks on MSBN media containing 100 μg/mL kanamycin (Rothstein et al., 1987b). Kanamycin-resistant shoots were placed on MS agar without hormones for root initiation. Between 6 and 12 kanamycin-resistant *N. sylvestris* or *N. tabacum* plants were selected for greenhouse propagation. They had been transformed with either pML507 for anionic peroxidase overproduction or pCib10 as a vector-only control. Seeds from self-fertilized transgenic plants were sowed in soil-less media and grown in the greenhouse with 14-hr day periods. Kanamycin-resistant progeny were selected by sowing surface-sterilized seeds on hormone-free media plus 100 μg/mL kanamycin.

Peroxidase Enzyme Assays and Isoelectric Focusing

The first two fully expanded leaves from equivalent greenhouse-grown tobacco plants were analyzed for peroxidase activity. Triplicate 500-mg samples of leaf tissue were homogenized on ice with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in three volumes of 10 mM sodium phosphate buffer (pH 6.0) and 1% sodium bisulfite as a reducing agent. The homogenate was centrifuged for 10 min at 14,000*g*, and the cleared supernatant was saved on ice for analysis. Total peroxidase activity was determined by the increase in absorbance at 470 nm in 0.28% guaiacol, 0.05 M sodium phosphate buffer (pH 6.0), and 0.3% H_2O_2 (Lagrimini and Rothstein, 1987). Fourteen-day-old seedlings from self-fertilized plants were assayed for peroxidase in the same manner; however, one cotyledon-derived leaf was removed and homogenized in a microcentrifuge tube with a pellet pestle (Kontes Scientific, Vineland, NJ). Protein concentration was determined by the Bradford reagent method (Bio-Rad, Richmond, CA). Protein concentration was found not to vary by more than 10% from 40 mg/g of tissue. The leaf extracts above were also subjected to analytical isoelectric focusing on flat bed polyacrylamide gels (pH 3.5 to 9.5) (Pharmacia LKB Biotechnology). Extract from 5 mg of leaf tissue was subjected to 0.125 W/cm^2 for 1.5 hr at 10°C. Gels were stained for peroxidase activity with 4-chloro-1-naphthol (Lagrimini and Rothstein, 1987).

RNA Isolation and Blot Hybridizations

Total RNA was isolated from young expanded tobacco leaves as described by Lagrimini et al. (1987). Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography (Maniatis, Fritsch, and Sambrook, 1982). Leaf poly(A)$^+$ RNA (1 μg) was first subjected to agarose-formaldehyde gel electrophoresis (Lehrach et al., 1977) and then transferred to a nylon filter (Hybond-N; Amersham, Arlington Heights, IL). The filters were air-dried briefly and then irradiated RNA-side up with ultraviolet light (256 nm) for 1 min (Khandjian, 1987). The filters were then hybridized with nick-translated $pPOD_{3.5}$, washed, and subjected to autoradiography as described by Thomas (1980).

Monitoring of Stomatal Aperture

Stomatal aperture and leaf conductance were monitored by two methods: direct examination of stomata under the microscope or leaf conductance as measured by a LI-6000 steady-state leaf porometer (Li-Cor Co., Lincoln, NE). These experiments used greenhouse-grown *N. tabacum* wild-type or 507C transformed plants 1 week after the onset of wilting. The epidermis was removed from plants in the early morning (6:00 AM to 8:00 AM) or mid-afternoon (12:00 PM to 2:00 PM) and immediately placed on microscope slices and covered with immersion oil. The immersion oil froze the stomata in position (Fisher, 1968). The stomata were examined and photographed under low magnification with a phase-contrast light microscope (Leitz). Measurements of leaf conductance were taken at several times during the same periods on three to five leaves from each of five identical F2 generation plants derived from self-fertilized seed as directed by the manufacturer.

Histology

Stem sections from 30-cm plants were fixed in formaldehyde:ethanol:acetic acid and embedded in parafin (O'Brien and McCully, 1981). Microtome cross-sections (13 μm) were stained with safrinin, methyl violet, fast green, and orange G. Xylem

vessels were extracted by hand and stained with phloroglucinol for longitudinal examination.

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