

# Inhibition of Phenolic Acid Metabolism Results in Precocious Cell Death and Altered Cell Morphology in Leaves of Transgenic Tobacco Plants

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Several complex phenotypic changes are induced when the transcription factor AmMYB308 is overexpressed in transgenic tobacco plants. We have previously shown that the primary effect of this transcription factor is to inhibit phenolic acid metabolism. In the plants that we produced, two morphological features were prominent: abnormal leaf palisade development and induction of premature cell death in mature leaves. Evidence from the analysis of these transgenic plants suggests that both changes resulted from the lack of phenolic intermediates. These results emphasize the importance of phenolic secondary metabolites in the normal growth and development of tobacco. We suggest that phenolic acid derivatives are important signaling molecules in the final stages of leaf palisade formation and that phenolic acid derivatives also play a prominent role in tissue senescence.

## INTRODUCTION

Secondary metabolites, by definition, serve no obvious function in general metabolism (Bu'Lock, 1961). A more contentious issue is whether they serve any function in the normal growth and development of the organisms that produce them. One view is that many secondary metabolic pathways have evolved as deterrents to potential predators or pathogens (Osborn, 1996), although it has been suggested that secondary metabolism has a primarily biochemical function, namely, relieving the accumulation of metabolic intermediates, maintaining nitrogen cycling, and maintaining the reducing potential of cells through oxidative reactions (Haslam, 1993).

The pathway of phenolic acid metabolism in plants requires the initial steps of general phenylpropanoid metabolism and provides the precursors for lignin biosynthesis. However, intermediates in the pathway and derivatives of these intermediates are ubiquitous in plants and accumulate to significant levels in tissues that do not synthesize lignin. The function of these intermediates, if any, is not yet clear, although several different roles have been proposed.

Phenolic compounds, particularly hydroxycinnamates, are present at significant levels in plant cell walls, where they may act as molecular bridges. For example, in grasses, 4-coumaric acid and ferulic acid link lignin to polysaccharide polymers, such as glucuronoarabinoxylan, through labile ester and/or ether bonds (Jung et al., 1993; Wallace and Fry, 1994). In the Chenopodiaceae, hydroxycinnamic acids are found in all cell walls attached to pectic polysaccharides (Wallace and Fry, 1994). Pectins may also be cross-linked to other pectins or to other noncellulosic polysaccharides through ester linkages with dimerized hydroxycinnamic acids, such as diferulic acid (Fry, 1986; Parr et al., 1996; Waldron et al., 1997). Although the importance of this cross-linking role has been difficult to evaluate (Fry, 1983), there is evidence that cross-linking of matrix polysaccharides through diferulic acid bridges in oats plays a protective role, because it increases in an incompatible interaction with the crown rust pathogen to provide a barrier to pathogen ingress (Ikegawa et al., 1996).

Because some phenolic acid derivatives can mimic the effects of cytokinins, they have also been implicated in the regulation of cell division and expansion. Two enantiomeric isomers (A and B) of dehydroadipic alcohol glucoside (DCG) have significant growth-promoting activities when used in tobacco pith or tobacco leaf bioassay systems (Binns et al., 1987). A third enantiomeric isomer (E) has somewhat lower activity (Lynn et al., 1987). In tobacco, DCGs are present both in quiescent tissue and in rapidly growing auxin- or cytokinin-stimulated tissues. In rapidly growing tissues, however, the levels of DCGs are approximately two

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orders of magnitude higher than in quiescent tissues, suggesting that growth regulators, such as cytokinins, may stimulate the accumulation or availability of DCGs, which in turn promote some of the physiological responses of plant cells to the growth regulators (Binns et al., 1987). Therefore, DCGs may act as components in a cytokinin-mediated regulatory cascade controlling cell division and possibly cell expansion (Teutonico et al., 1991).

Hydroxycinnamic acid esters also serve as antioxidants *in vitro*, and it has been suggested that *in vivo* they may serve as natural antioxidants for lipids (Daniels and Martin, 1961, 1964, 1967; Daniels et al., 1963; Rice-Evans et al., 1997). Although more attention has been paid to flavonoids as potential natural antioxidants (Rice-Evans et al., 1997; Yamasaki, 1997), phenolic acid esters, such as chlorogenic acid, also perform well as antioxidants, particularly in protecting lipids from peroxidation (Rice-Evans et al., 1997).

In summary, intermediates in phenolic acid metabolism and monolignol synthesis may have a variety of functions throughout the plant, namely, protecting it from pathogen and/or predator attack, providing stabilizing cell wall cross-links, providing signal molecules to regulate cell division and expansion, and possibly playing a role as natural antioxidants.

A classic way to address the function of a pathway or compound is to examine the effects of its loss on the form and function of the affected organism. We have analyzed transgenic tobacco plants inhibited in phenolic acid metabolism and monolignol biosynthesis (Tamagnone et al., 1998) to investigate whether intermediates in this branch of secondary metabolism play a significant part in the growth and development of tobacco leaves. One aspect of the phenotype that is invoked by overexpression of *AmMYB308*, a gene encoding a MYB transcription factor from *Antirrhinum*, is reduced lignin biosynthesis, as predicted from the down-regulation of the biosynthetic pathway (Tamagnone et al., 1998). However, two other important aspects of the phenotype created by overexpression of *AmMYB308* cannot be explained by the inhibition of lignin production. These are a modification of leaf palisade cell development and an abnormal and premature cell death invoked when the leaf matures. Analysis of these phenotypic traits provides evidence for the important roles played by the derivatives of phenolic acid metabolism in regulating these aspects of normal growth and development in tobacco.

## RESULTS

### Effect of *AmMYB308* Overexpression on Leaf Cell Development

Overexpression of the *AmMYB308* gene from *Antirrhinum* in tobacco affected the growth of most organs of these plants

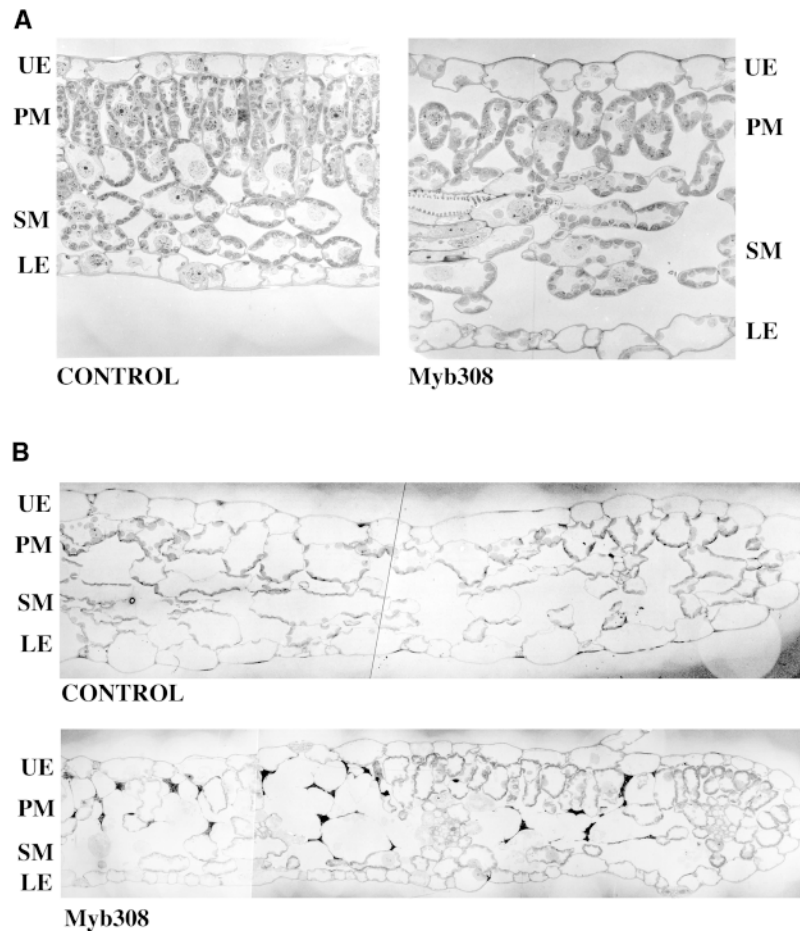
(termed Myb308 plants), but an effect observed very early in development was that the leaves had pale pigmentation (Tamagnone et al., 1998). Cotyledons of Myb308 seedlings appeared pale green, especially in contrast to residual darker green tissue around the veins. This pale green phenotype was maintained as the leaves matured. Leaf sections were prepared from young (2 cm long), intermediate (~10 cm long; almost fully expanded), and older leaves (fully expanded with small lesions starting to appear) and were compared with leaf sections from untransformed Samsun plants (controls) of the same age (Figure 1).

Young leaves from both Myb308 and control plants were basically similar, with tightly packed cells in the mesophyll. In intermediate-aged leaves, differences in the palisade cells were apparent (Figure 1A). In controls, the palisade mesophyll could be distinguished as a layer of closely packed, columnar cells beneath the upper epidermis. In Myb308 plants, the palisade cells were more isodiametric, with large air spaces between them, suggesting reduced cell-to-cell adhesion.

Striking differences were also observed in sections of older leaves (Figure 1B). In control leaves in which senescence was beginning, the cells were highly vacuolate, showing loss of cell contents, and the cell walls appeared fluted, arching inward at positions adjacent to chloroplasts. However, nuclei and identifiable organelles could still be detected. In Myb308 leaves, the cells did not appear to senesce normally. Rather, among cells that appeared morphologically normal, there were isolated dead cells that were unusual in appearance: collapsed, avacuolate, and very electron dense. Such isolated dying and dead cells were located largely in the spongy and palisade layers of the mesophyll. Dead, collapsed cells of this type were never observed in control sections. No viral particles or bacterial cells were ever seen associated with the dead cells in Myb308 plants.

### Form of the Dead Cells in Myb308 Leaves

The cellular contents of the dead cells in Myb308 leaves were very electron dense, although the remains of thylakoid membranes could be distinguished (Figure 2A). The nuclei were also smaller than in healthy cells, and they contained highly condensed chromatin. Sections from older Myb308 leaves showed some cells in a more intermediate stage of collapse, with highly condensed nuclei and expanded chloroplasts (Figure 2A; lower cell). The cytoplasmic contents had precipitated or condensed to form darkly staining amorphous material, and the cells were avacuolate. In some cells surrounding dying or dead cells, the chloroplasts appeared swollen (Figure 2B, cell type II); this could represent an early stage in cell death because otherwise their cytoplasmic integrity appeared normal. The general appearance (corpse morphology) was similar to that described in animal cells as apoptotic (Wyllie et al., 1980), because death occurred initially in isolated cells and involved inward cellular collapse



**Figure 1.** Electron Microscopic Analysis of Leaves from Untransformed Control Plants and Myb308 Plants.

**(A)** Transverse sections of mature leaves (10 cm long). The palisade layer of the Myb308 leaves (right) contained rounded cells lacking the packed columnar form seen in control sections (left). The larger air spaces between the Myb308 cells probably resulted from decreased cell-to-cell adhesion, which caused the tissue to separate during fixation.

**(B)** Sections of older leaves. In control leaves (top), the senescing cells were highly vacuolate with fluted cell walls. In Myb308 leaves (bottom), isolated dead cells are visible in both the palisade and spongy mesophyll as dark electron-dense corpses.

LE, lower epidermis; PM, palisade mesophyll; SM, spongy mesophyll; UE, upper epidermis.

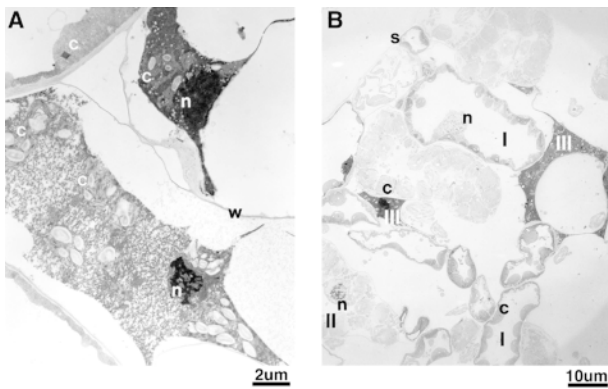
and condensation of chromatin to give granular aggregates in small condensed or pycnotic nuclei.

#### Modification of Cell Shape and Number in Myb308 Leaves

To quantify the differences in the palisade cells of Myb308 leaves, we took measurements of cell width, height, section area, and perimeter from a series of scanning electron micrographs (Figures 3A and 3B) of control and Myb308 leaves of intermediate age after freeze fracture. In these micrographs, the differences in size and shape of palisade cells were the same as those observed in the transmission

electron micrographs; however, the large air spaces observed in transmission electron microscopic sections of Myb308 plants were not observed in similar sections prepared by freeze fracture. It is likely that these large air spaces arose as an artifact during tissue fixation, because it involved vacuum infiltration; cells in Myb308 leaves presumably were more susceptible to the effects of the vacuum (compared with palisade cells of control leaves) and were pulled apart during fixation.

In rapidly frozen and fractured leaves, the maximum cell section area, the maximum cell perimeter, and the mean length of the short axis of the palisade cells were all greater in Myb308 leaves than in control leaves. The long axis was comparable between the palisade cells of the two lines (Table 1).



**Figure 2.** Details of Cell Death in Myb308 Plants.

**(A)** Dead cells showing condensed nuclear material (n). The cytoplasmic contents appear to be aggregated, and the position of the chloroplasts (c) can be distinguished by their thylakoid membranes and starch granules. The vacuole is absent, and the condensed cytoplasmic contents have separated from the cell walls (w). The upper cell is in a more advanced stage of collapse than is the lower cell. **(B)** Intermediate stages in cell death in Myb308 leaves. Three types of cells are apparent: type I cells are normal with crescent-shaped plastids (c) and normal nuclei (n); type II cells have inflated chloroplasts and condensed chromatin in their nuclei, suggesting that these cells may be at an intermediate stage between cell type I and cell type III, which are dead. All three cell types can be found together, illustrating a degree of cell autonomy for the processes of cell death. A stoma (s) is also shown.

This confirmed our observation that Myb308 palisade cells were wider and rounder than wild-type columnar palisade cells. The mean values for the long and short axes of Myb308 and wild-type cells were used to calculate the average cell surface area and volume, assuming that the control cells were cylindrical and that the Myb308 cells were prolate spheroids. These values are listed in Table 2. Although the differences in surface area between the two cell types were not striking, the volume of Myb308 cells was considerably greater than that of control cells.

In sections of a given length, there were also therefore fewer palisade cells in Myb308 leaves than in control leaves (an average of 30% fewer cells), as shown in Table 3. Myb308 leaves were smaller than control leaves at maturity, emphasizing that *AmMYB308* has a significant effect on palisade cell number in leaves as well as on cell shape.

#### Gravimetric Analysis of Myb308 and Control Leaves

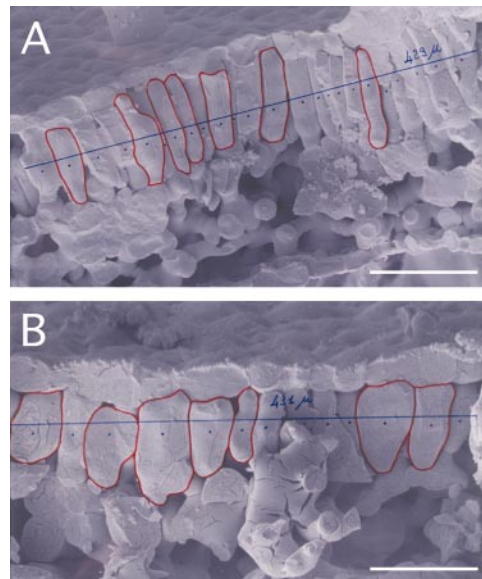
Thin sections of leaves suggested that cell wall production in Myb308 palisade cells may be limited. We estimated the effect of *AmMYB308* on cell wall production by gravimetric analysis of fully expanded leaves because, developmentally,

these probably represent the end of net wall deposition during leaf development.

Given an equal amount of fresh leaf material, Myb308 samples contained, on average, 39% less total dry weight than did the controls (Table 4). Most of the loss of dry weight was due to a reduction (48%) in insoluble material representing cell wall material. There was a much smaller reduction in soluble material (25%). Clearly, such a significant decrease in cell wall material cannot be explained solely as a result of reduced cell wall phenolics, especially in the predominantly nonlignified tissue of the leaf blade.

#### Suspension Cultures of Myb308 and Control Tobacco Cells

To determine the cause of the altered growth of Myb308 palisade cells, we tried to feed phenolic acid intermediates to Myb308 plants, but it was not possible to feed them to intact plants reliably, so cell suspension cultures were established from Myb308 and control plants (untransformed or transformed with binary vector T-DNA alone). These cul-



**Figure 3.** Scanning Electron Microscopy of Freeze Fracture Sections of Mature Control and Myb308 Leaves.

**(A)** Control section.

**(B)** Myb308 leaf section.

To measure the maximum perimeter, maximum section area, long axis, and short axis, cells of the palisade that stood out on the photograph and whose edges were clearly visible were circled and measured with a computerized graphics tablet. The sections were also used to count the number of cells in a given length of leaf section, as shown in **(A)** and **(B)**. Bars = 100  $\mu$ m.

**Table 1.** Measurements of Leaf Palisade Cells

Leaf Sample <sup>a</sup>	Maximum Section Area		Maximum Perimeter		Long Axis		Short Axis	
	<i>n</i> <sup>b</sup>	( $\mu\text{m}^2$ ) <sup>c</sup>	<i>n</i>	( $\mu\text{m}$ ) <sup>c</sup>	<i>n</i>	( $\mu\text{m}$ )	<i>n</i>	( $\mu\text{m}$ )
Control (YL)	30	423 $\pm$ 18	30	94 $\pm$ 2	31	38 $\pm$ 1	31	12 $\pm$ 1
Myb308 (YL)	62	661 $\pm$ 17	62	108 $\pm$ 1	65	37 $\pm$ 1	65	20 $\pm$ 1
Control (IL)	62	1470 $\pm$ 35	62	190 $\pm$ 3	64	74 $\pm$ 1	64	20 $\pm$ 1
Myb308 (IL)	47	2930 $\pm$ 112	47	242 $\pm$ 6	47	86 $\pm$ 2	47	39 $\pm$ 1
Control (ML)	46	4901 $\pm$ 155	46	355 $\pm$ 6	48	138 $\pm$ 2	48	37 $\pm$ 1
Myb308 (ML)	27	5854 $\pm$ 234	27	351 $\pm$ 9	27	128 $\pm$ 4	27	56 $\pm$ 2

<sup>a</sup>YL, young leaves; IL, intermediate leaves; ML, mature leaves.

<sup>b</sup>*n*, the number of sections analyzed.

<sup>c</sup>Mean area ( $\mu\text{m}^2$ ) or dimension ( $\mu\text{m}$ )  $\pm$  SD.

tured cells have been developed to model the growth of palisade cells (Stickens et al., 1996; Tao and Verbelen, 1996), especially developing palisade cells that are not fully autotrophic but are dependent on both light and imported sucrose, as are the cultured cells.

Myb308 and control cell cultures had quite distinct appearances when viewed under the light microscope (Figure 4). Cell cultures derived from wild-type plants or plants transformed with the binary vector (pBin19) alone were indistinguishable. Both consisted of finely dispersed cell suspensions; the cells were elongated, rod shaped, and either straight, bent, or twisted in spirals (Figure 4A). Cells in the Myb308 cultures consisted predominantly of aggregates with a few dispersed cells. The aggregates ranged from a few to many hundreds of cells. The cells themselves were box shaped and very distinct from the control cells (Figure 4B). We examined the ability of the cell cultures to divide by counting cells at defined times after subculturing. Both cultures showed a lag phase followed by an increase in cell number after 4 days. However, the rate of increase of control cultures was greater than for Myb308 cultures, with estimated doubling times of 1.7 and 2.1 days, respectively (Figure 5A). These results were reproducible for two independently derived control cultures and three independently derived Myb308 cultures. These measurements suggested that the cell cultures reflected the different behavior observed in the palisade cells of control and Myb308 plants.

### Feeding Cell Cultures with Phenolic Precursors of Plant Growth Factors

Because Myb308 plants are deficient in phenolic intermediates (Tamagnone et al., 1998), it is possible that their limited cell wall formation and cell division/expansion might be due to their inability to produce phenolic-based growth factors. Among the phenolic derivatives with growth-promoting activity are the DCGs. To test whether the alterations in cell growth might result from deficiencies in endogenous DCGs,

we measured the levels of the active DCG isomers in Myb308 and control plantlets grown in culture by methanol extraction and HPLC analysis (Figure 6A). DCGs were readily detected in control plants (0.33 nmol/g fresh weight of active isomers A and B and 0.83 nmol/g fresh weight of another isomer[s] tentatively identified from its UV spectrum as DCG D and/or E); however, DCG isomers A and B could barely be detected in Myb308 plants (<0.14 nmol/g fresh weight), and isomers D and E were reduced by at least 80% (<0.19 nmol/g fresh weight). The aglycone dehydrodiconiferyl alcohol (DCA) was undetectable in either control or Myb308 plants. We repeated these assays with maturing leaf tissue from independent plants. DCA was undetectable in control and Myb308 leaves. The active DCG isomers (A and B) were measured at 7.1 nmol/g fresh weight, and the other isomers (D and E) were measured at 1.6 nmol/g fresh weight in control leaves. Only the active isomers (A and B) were detected in Myb308 leaves and were measured at 0.16 nmol/g fresh weight (Figure 6B).

To determine whether the alterations in the growth of Myb308 cells resulted from deficiencies in the levels of DCGs, we then prepared the aglycone precursor DCA and fed it to control and Myb308 cell cultures to test its effects on cell growth. The enzymatic production of DCA yielded three isomers. The most abundant was 5,β'-dehydrodiconiferyl alcohol (~50% of the products), which is the aglycone

**Table 2.** Average Surface Area and Volume of Leaf Palisade Cells

Leaf Sample <sup>a</sup>	Surface Area ( $\mu\text{m}^2$ )	Volume ( $\mu\text{m}^3$ )	Volume/Area
Control (YL)	1,672	4,363	2.6
Myb308 (YL)	2,053	7,992	3.9
Control (IL)	5,191	22,629	4.4
Myb308 (IL)	8,840	67,621	7.6
Control (ML)	18,035	145,912	8.1
Myb308 (ML)	18,997	211,207	11.1

<sup>a</sup>YL, young leaves; IL, intermediate leaves; ML, mature leaves.

**Table 3.** Number of Cells in Palisade Mesophyll

Leaf Sample <sup>a</sup>	Average Number of Cells per mm <sup>2</sup>
Control (YL)	8280
Myb308 (YL)	4360
Control (IL)	3364
Myb308 (IL)	1225
Control (ML)	784
Myb308 (ML)	576

<sup>a</sup>YL, young leaves; IL, intermediate leaves; ML, mature leaves.

of the active DCGs. HPLC analysis of methanolic extracts of Myb308 cells that were fed DCA showed that intracellular DCA levels increased, as did the levels of various DCG isomers, including the most active forms of DCG, isomers A and B (Figures 7A and 7B). The Myb308 cells therefore appeared to be able to convert DCA to DCGs. HPLC analysis showed not only the formation of different DCG isomers within the cells but also the limited metabolism of DCA or DCG to other phenolics in the cultures, which is consistent with previous results showing slow turnover of DCGs (Orr and Lynn, 1992). In contrast, when a mixture of DCG A plus B was fed to cells, the DCGs were not readily taken up and remained instead in the culture medium (data not shown).

Treatment of control cells with DCA produced no obvious visible changes in their appearance. However, DCA had a dramatic effect on the appearance of Myb308 cells (Figures 8A to 8D). After 4 days in the medium with DCA, some of the cells started to elongate, and the culture as a whole became more dispersed, starting to resemble a control culture. These changes peaked at day 7, when a large proportion of the cells were elongated and cell aggregates were greatly reduced in number and in size. After 1 week, the effect began to decline, although the cultures retained their altered appearance up to day 16.

This experiment was repeated four times with DCA from different sources with consistent results. By counting cells, we also measured the effects of DCA on the rates of division of control and Myb308 cells. Treatment of control cultures with DCA had no effect on cell proliferation (Figure 5B). Untreated Myb308 cells again showed reduced rates of proliferation compared with controls. The treatment of two independent Myb308 cultures with DCA increased proliferation rates to levels comparable with those of the control cultures (Figure 5C).

These results support the view that the abnormal growth and division of leaf palisade cells in Myb308 plants might have the same cause as the abnormal growth in the suspension cultures and result from lower levels of phenolic acid derivatives. The particular derivatives involved were probably the isomers of DCG. Presumably, the consequences of a reduction in DCG levels are most marked in leaf palisade cells, because these have specific requirements for such

growth signals in developing their normal, tightly packed columnar form in the final stages of leaf development. Interestingly, the levels of DCGs measured in maturing leaves of control plants were >10-fold greater than those measured for whole plantlets, indicating particularly high DCG accumulation in maturing leaves.

The altered shape of the palisade cells and the reduced cell number in Myb308 leaves, which we believe, from these results, to be due to a lack of phenolic signaling compounds, mean that the chloroplasts are not as densely packed one above the other in the cells as they are in the normal columnar cells of control leaves. This may explain the paler appearance of Myb308 leaves.

### Premature Cell Death in Myb308 Leaves

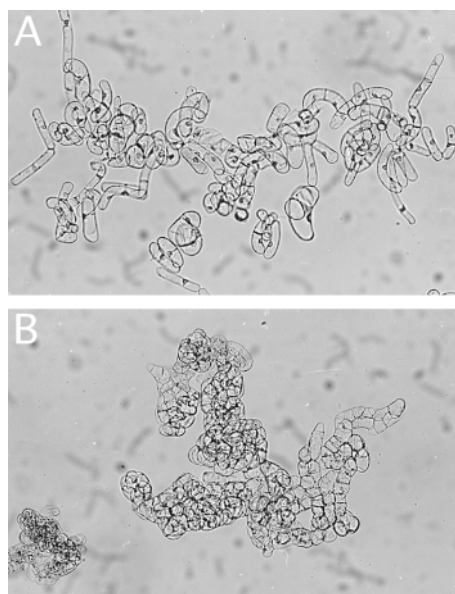
The other prominent effect of *AmMYB308* overexpression was the premature induction of cell death in mature leaves, similar to apoptotic cells described for animals by Wyllie et al. (1980). A characteristic event in many apoptotic animal cell deaths is the breakdown of nuclear DNA by endonucleolytic cleavage. A sensitive method for detecting nicking of DNA is in situ tagging of the 3' hydroxyl ends of DNA generated during endonucleolytic DNA fragmentation (TUNEL labeling; Gavrieli et al., 1992).

To investigate this cellular collapse, we compared sections of mature leaves from Myb308 and control plants. In control plants, very few nuclei were stained (Figure 9A), and these were almost exclusively restricted to differentiating xylem elements (Figure 9B), similar to results reported by Mittler and Lam (1995). In Myb308 leaves, in contrast, many nuclei were stained. These were dispersed in a scattered fashion and present in all tissues of the leaf, including vascular tissue (Figure 9C) and mesophyll and epidermal cells (Figure 9D). The nuclei stained in cells before their collapse (Figures 9D and 9E).

**Table 4.** Gravimetric Analysis of Fully Expanded Leaves from Myb308 and Control Tobacco Plants

Sample	Dry Weight <sup>a</sup>			
	Total Dry Weight	Cell Wall Material	Soluble Material	Estimated Recovery (%)
Control leaves	14.0 ± 1.2	7.8 ± 1.3	4.4 ± 0.5	87.0 ± 5.2
Myb308 leaves	8.6 ± 0.8	4.0 ± 0.7	3.3 ± 0.7	85.2 ± 7.0

<sup>a</sup>Means and standard deviations of three control lines and six Myb308 lines derived from four independent primary transformants are shown. Measurements are given as the percentage of fresh weight of tissue.



**Figure 4.** Comparison of the Appearance of Suspension Culture Cells from Control and Myb308 Tobacco Plants.

(A) Cells from a control culture transformed with pBin19 alone.

(B) Cells from a Myb308 culture.

Control cells typically had an elongated rod shape and were relatively dispersed, whereas Myb308 cells were box shaped and tended to form large aggregates.

#### Effects of AmMYB308 on Malondialdehyde Levels

The premature and abnormal cell death in the Myb308 plants could result from increased oxidative stress, because phenolic acid esters are excellent antioxidants (Daniels and Martin, 1961, 1964, 1967; Daniels et al., 1963). The deficiency of such natural antioxidants in Myb308 plants could lead to an increase in the concentration of reactive oxygen species (ROS) above the threshold necessary to trigger cell death. This might be particularly significant in mature and senescing leaves, in which increases in the production of  $O_2^-$  and  $H_2O_2$  have been demonstrated (Thompson et al., 1987; Pastori and del Rio, 1997). Lipid peroxidation, which arises from increased ROS, has been shown to increase in tobacco leaves from the time that they reach maturity (Dhindsa et al., 1981). A by-product of lipid peroxidation is malondialdehyde (MDA), and measurement of MDA levels in cells can be used to assess the extent of damage to membrane lipids (Dhindsa et al., 1981).

We assayed the levels of MDA in two independent experiments. In each experiment, fully expanded leaves of three control and three Myb308 plants were compared for the amount of MDA per milligram of protein. The results, summarized in Table 5, show that Myb308 leaves have double the MDA levels of control leaves of the same age.

The morphology of the dead and dying cells in Myb308 leaves is very similar to that of cells that have died as part of a hypersensitive response (HR) to an incompatible plant pathogen. An early component of the HR may be the oxidative burst generated in response to an avirulent pathogen (Doke and Ohashi, 1988; Chen et al., 1993; Legendre et al., 1993; Draper, 1997). The association of cell death with the production of ROS in both cases may account for the morphological similarities between dead cells resulting from the HR and the dead cells of Myb308 leaves.

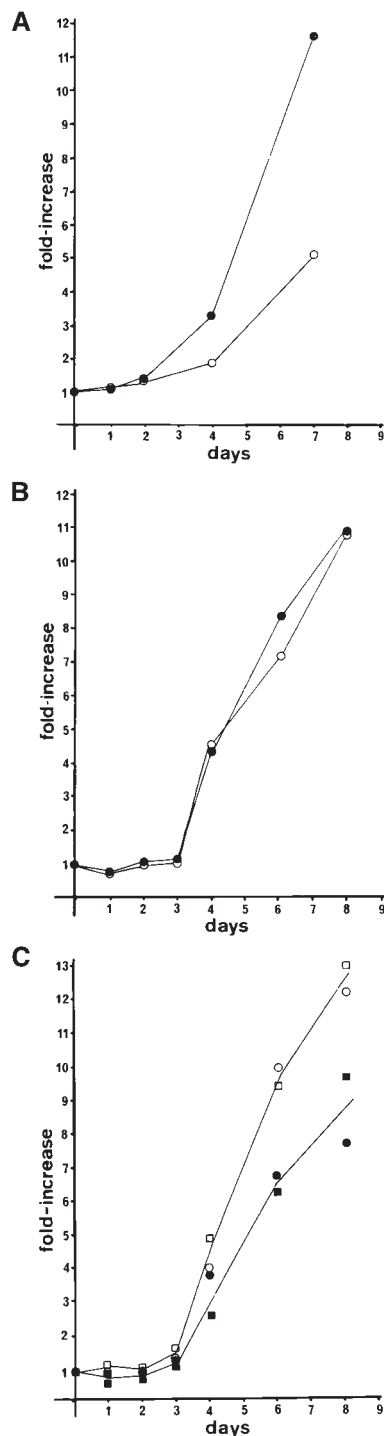
To test the effect of lowered phenolic production on the dynamics of the HR, we compared the kinetics of the HR in Myb308 and control plants by using the M2 strain of *Pseudomonas syringae* pv *maculicola*. The development of the HR was monitored visually to determine the responding leaf area and quantified by measuring ion leakage from 7-mm discs cored from infected, water-flooded, and untreated areas of leaves at successive time points.

Visual observation indicated that the kinetics of the HR were different in Myb308 and control plants. The onset of the HR was always earlier in Myb308 plants; visible symptoms (loss of turgor and a silvery sheen to the lower leaf epidermis) appeared between 1 and 5 hr after infection compared with symptoms appearing between 2 and 5 hr after infection in control plants. The final extent of HR in terms of the area of the leaf affected, however, was the same in Myb308 and control leaves. Ion leakage, measured as conductivity, also showed that the HR proceeds more rapidly in Myb308 than in control plants, although it was possible to sample only a few time points to avoid extensive mechanical damage to the leaves (Figure 10).

These results support the view that the absence of phenolics decreases the ability of Myb308 plants to withstand ROS. Although an avirulent pathogen (presumably inducing an oxidative burst) was still required to induce the HR in Myb308 plants, the response was induced more rapidly. We were unable to induce the HR with nonpathogens, such as *Escherichia coli*, however.

#### Effect of Salicylic Acid on the Myb308 Phenotype

A block in phenylpropanoid metabolism after the step catalyzed by phenylalanine ammonia-lyase (Tamagnone et al., 1998) could lead to a buildup of cinnamate, resulting in increased synthesis of salicylic acid (SA) that might contribute to the formation of the HR-like lesions on the leaves of Myb308 plants, perhaps by inducing the synthesis of ROS (Draper, 1997). SA is also involved in the systemic acquired resistance of plants to pathogens (Dempsey and Klessig, 1994). To ascertain the contribution of SA to the Myb308 phenotype, we crossed Myb308 plants to a homozygous tobacco line constitutively expressing the *nahG* gene from *Pseudomonas putida* (a gift of J. Draper; Bi et al., 1995). The *nahG* gene encodes the enzyme salicylate hydroxylase that converts SA to catechol, a compound that does not induce



**Figure 5.** Relative Growth Rates of Control and Myb308 Cultures and the Effects of DCA on Culture Growth.

**(A)** Relative growth rates of control (filled circles) and Myb308 (open circles) cultured cells. The fold increase in cell number is shown against the time from subculture in days.

systemic acquired resistance. If the precocious cell death observed in Myb308 plants were the consequence of a high SA concentration, hybrid plants should not show this aspect of the Myb308 phenotype, because the presence of salicylate hydroxylase would keep SA levels very low. On the other hand, if the phenotype were still to persist in the hybrid plants, it would be unlikely that SA is involved in the precocious cell death. We found that progeny from the cross still showed the typical Myb308 phenotype, including cell death in leaves (Figure 11), ruling out SA as the cause of the lesions and supporting the hypothesis that increased oxidative stress, resulting from the lack of ROS-buffering phenolics, is the trigger of the cell death process.

## DISCUSSION

Tobacco plants in which phenolic acid (and monolignol) biosynthesis has been inhibited by the Antirrhinum transcription factor AmMYB308 (Tamagnone et al., 1998) provide experimental material with which to evaluate the importance of this branch of secondary metabolism in the normal growth and development of plants. The only significant and consistent change in gene expression that we have found in Myb308 plants is reduced steady state levels of transcripts of those genes involved in hydroxycinnamic acid and monolignol metabolism (Tamagnone et al., 1998). In the Myb308 tobacco lines, in addition to a reduction in lignin biosynthesis, two other marked changes in the phenotype were observed: leaves were paler than those of control plants, and cells in mature leaves undertook precocious cell death. A very similar phenotype has been reported for tobacco plants showing reduced activity of phenylalanine ammonia-lyase and, consequently, reduced hydroxycinnamic acid/monolignol metabolism (Elkind et al., 1990). This suggests that paler leaves and premature leaf cell death arise as a direct consequence of reduced phenolic acid metabolism.

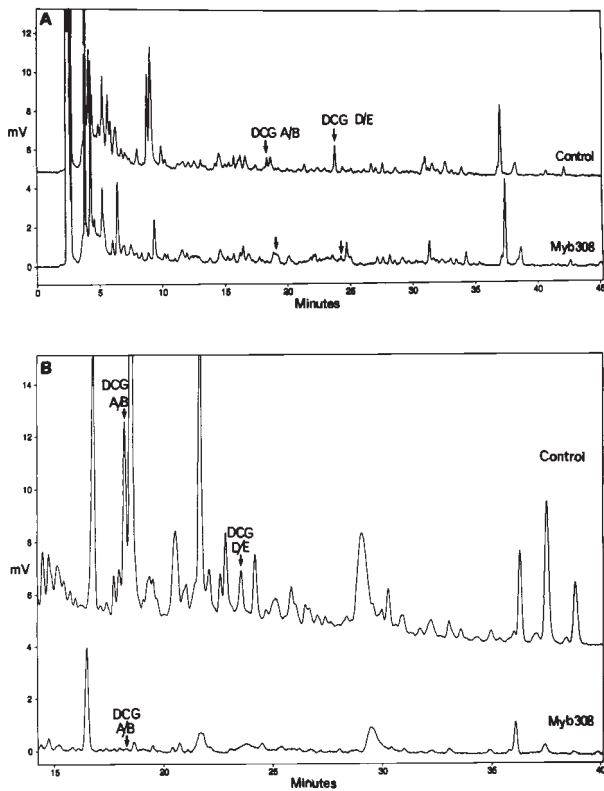
### Phenolic Acids and the Control of Palisade Cell Development

The marked alteration in palisade cell shape in Myb308 plants appeared to involve the accumulation of reduced cell wall material. This is supported by ultrastructural analysis of

**(B)** Relative growth rates of control cells untreated (filled circles) or supplemented with DCA in the media (open circles).

**(C)** Relative growth rates of Myb308 cells untreated (filled circles, one culture line; filled squares, second independent culture line) and supplemented with DCA in the media (open circles represent the first culture line treated with DCA; open squares represent the second culture line treated with DCA).





**Figure 6.** Levels of DCA and DCG Isomers in Control and Myb308 Plantlets and Leaves.

**(A)** HPLC analysis of DCG content of control and Myb308 plantlets. Twenty-five microliters of methanolic extract was analyzed on a Columbus C18 reverse phase column (Phenomenex Ltd., Macclesfield, UK) with gradients of 90% solvent A and 10% solvent B at time 0, 60% solvent A and 40% solvent B at 50 min, 30% solvent A and 70% solvent B at 60 min, and 90% solvent A and 10% solvent B at 65 min, where solvent A was 1 mM trifluoroacetic acid and solvent B was acetonitrile. Absorbance was measured at 275 nm, and peaks were identified by reference to standard preparations of DCGs.

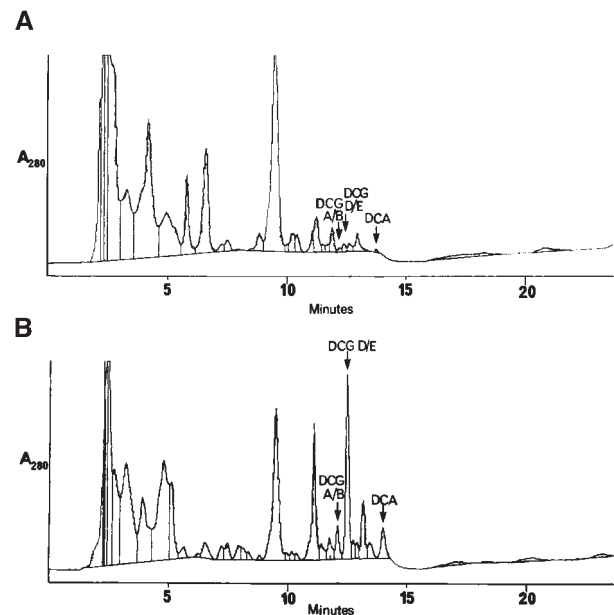
**(B)** HPLC analysis of DCG content in maturing control and Myb308 leaves. Methanolic extract (37.5  $\mu$ L) was analyzed as described above.

The peaks corresponding to DCG isomers A and B (A/B) and D and E (D/E) are indicated with arrows.

palisade cells but was not readily apparent for cells of the spongy mesophyll or epidermal tissues. Our failure to detect changes in cell shape or number in the spongy mesophyll may have been due to difficulties in recognizing such changes in cells of irregular form, and it is quite possible that the block in phenolic acid metabolism also affects the growth and development of the spongy mesophyll. Leaf palisade cells have relatively high requirements for cell wall production to achieve their normal columnar cell shape, and palisade cells may use specific signals for their final stages of differentiation and

morphogenesis. The recent description of genes involved in chloroplast differentiation that also specifically affect palisade development in leaves supports this view (Chatterjee et al., 1996; Keddie et al., 1996). There is also a reduction in cell number in Myb308 leaves as well as a modification of cell shape. We have used suspension cultures derived from the Myb308 and control plants to analyze the effects of inhibition of phenolic acid metabolism on cell growth and division. The results from the suspension cultures have to be interpreted with caution, but so far as can be determined, they reflect the differences observed in the palisade cells, including effects on cell elongation and reduced rates of cell division.

DCG is one candidate that may play a role in developmental signaling, because it has been shown to promote cell division



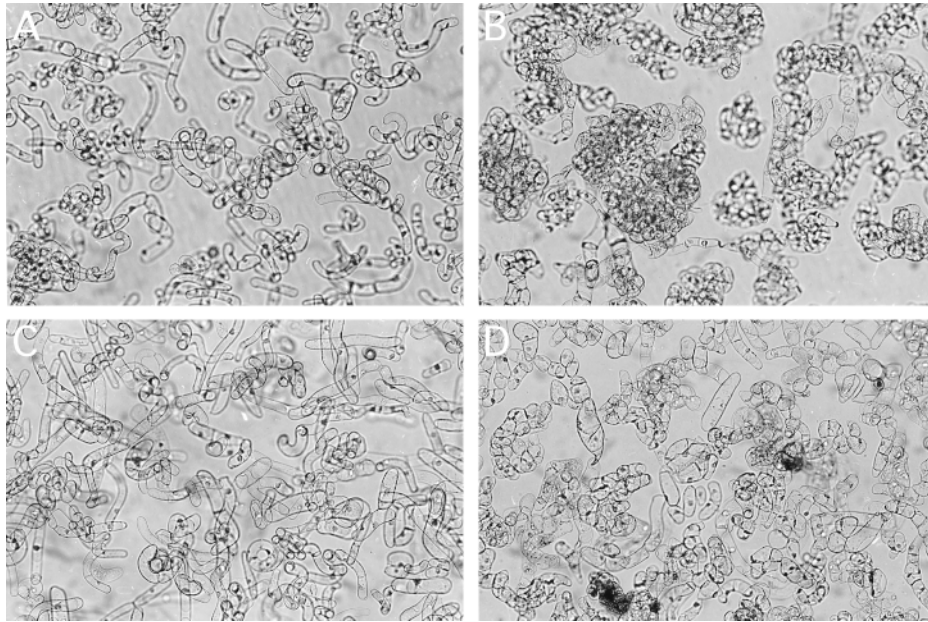
**Figure 7.** Levels of DCA and DCG Isomers in Untreated Myb308 Cultures and in Cultures Fed with Exogenous DCA.

Shown are the results of an analysis of DCG and DCA content of Myb308 suspension cultures grown in normal media and in media supplemented with 100  $\mu$ M DCA. Methanolic extracts of cells were run on a more compressed linear gradient (25 min) in conjunction with an Inertpak ODS2 1k5 column (Capital HPLC, Broxburn, UK). Solvent A was 10% MeOH, 90% (v/v) H<sub>2</sub>O, and 1 mM trifluoroacetic acid; solvent B was 80% MeOH, 20% (v/v) H<sub>2</sub>O, and 1 mM trifluoroacetic acid. The gradient was 90% solvent A and 10% solvent B at time 0, and 10% solvent A and 90% solvent B at 25 min.

**(A)** Myb308 cultures contained virtually undetectable levels of DCGs or DCA.

**(B)** Feeding DCA led to an increase in endogenous DCA but a much larger increase in endogenous DCGs, which was identified by reference to standard spectra.

The peaks corresponding to DCG isomers A and B (A/B) and D and E (D/E) and to DCA are indicated with arrows.



**Figure 8.** Effect of DCA on Growth of Suspension Cultures of Control and Myb308 Cells.

- (A) Control cells.  
 (B) Myb308 cells.  
 (C) Control cells plus 100  $\mu\text{M}$  DCA.  
 (D) Myb308 cells plus 100  $\mu\text{M}$  DCA.

Treatment with DCA had little effect on control cultures but induced the Myb308 cells to separate and elongate and to resemble control cultures (cf. [B] and [D]).

and cell expansion in tobacco (Binns et al., 1987; Lynn et al., 1987; Teutonico et al., 1991). Experiments feeding the aglycone of DCGs, DCA, to suspension culture cells demonstrated that DCA could be used by Myb308 cells to form several DCG isomers, including the biologically active forms. DCA could also restore the normal growth phenotype and rates of division to Myb308 cells in culture when supplied at 100  $\mu\text{M}$ . These data support a role for DCGs in tobacco cell differentiation in vivo and show that the synthesis of DCG is an important function of the phenolic acid/monolignol pathway in normal plant development.

The levels of DCA required to rescue the growth phenotype of Myb308 cultured cells were within the range used by others to promote cell expansion and division. Those levels are high relative to requirements for growth factors, such as cytokinins, but this may partly reflect the instability of DCA in solution.

### Phenolic Acids and the Control of Leaf Senescence

The second major impact of *AmMYB308* overexpression was observed during the senescence of tobacco leaves.

The occurrence of isolated dead cells coincided with the maturation of leaves and indicated a role for phenolic acid metabolism in tempering or buffering changes induced during senescence. Cell death had all of the morphological characteristics of apoptosis, but all of these features are also characteristic of cells that die as a result of oxidative damage. It is difficult to determine whether the cell death observed in Myb308 plants resulted from the direct effect of elevated ROS or from a central programmed cell death pathway that could be triggered by several of the signaling inputs, including elevated levels of ROS (Korsmeyer, 1995; Heath, 1998). Our results do, however, indicate a significant role for phenolics in inhibiting cell death during the normal controlled processes of leaf senescence.

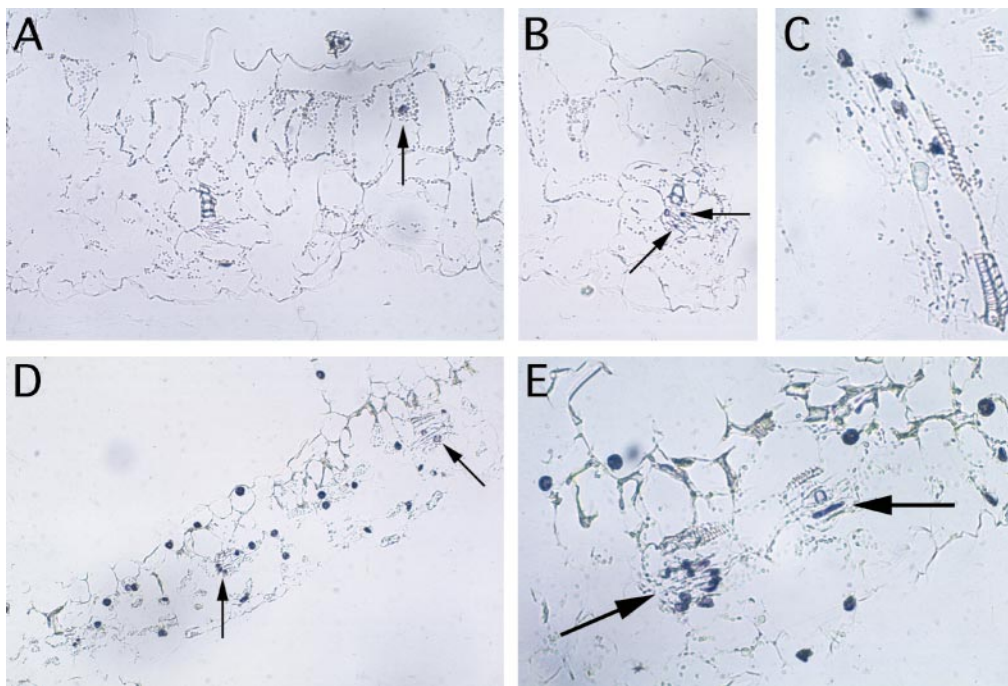
The cell death observed in the mature Myb308 leaves is likely to be a downstream consequence of the loss of phenolics. Oxidation of phenolics to *o*-quinones is triggered by membrane damage during senescence. Membrane damage releases lipoxygenase, which oxidizes membrane lipids (Thompson et al., 1987), and polyphenol oxidases from the plastids, which oxidize cellular phenolics. Membrane damage may also stimulate oxidation of phenolics in cell walls by peroxidases (Noodén et al., 1997). All of these changes re-

sult in the familiar browning of senescent tissues. Myb308 plants do not brown in this way but undergo an earlier death of individual cells, which results in leaf tissue eventually becoming completely dead and white (Tamagnone et al., 1998).

The results from the Myb308 plants suggest that oxidation of phenolics during senescence is part of a protective mechanism that reduces the impact of elevated ROS levels and allows for the gradual withdrawal of cellular material from aging leaves through controlled senescence. Supporting this explanation is recent enzymic evidence to suggest a role for chlorogenic acid in scavenging of hydrogen peroxide after cell damage (Takahama and Oniki, 1997). In the absence of antioxidant phenolics (either soluble in the vacuole or bound to the cell wall), the cells of aging leaves are not adequately protected against the oxidative stresses generated by the processes of senescence and die rapidly. This idea is supported by levels of lipid peroxidation in mature Myb308 leaves almost twice control values. Although we cannot attribute the cell death observed in Myb308 leaves

directly to activation of a programmed pathway, the morphology of cells was remarkably similar to apoptotic cells in animals. This was in contrast to the senescent cells of control leaves, which undoubtedly die in a programmed manner (Noodén et al., 1997; Pennell and Lamb, 1997). Some types of programmed cell death in plants, such as senescence, may therefore involve corpse morphologies distinct from apoptotic cells (Noodén et al., 1997; Heath, 1998).

One of the developmental changes believed to be closely analogous to apoptosis in animal cells is the HR of plants to avirulent pathogens (Heath, 1998). The HR involves an early oxidative burst and increased production of ROS, although it is not clear whether the biphasic oxidative burst observed in response to incompatible pathogens is sufficient in itself to cause cell death (Levine et al., 1994; Glazener et al., 1996; Lamb and Dixon, 1997). The HR to an incompatible strain of *P. syringae* was potentiated in Myb308 plants, compared with controls. These results are consistent with Myb308 plants having higher levels of ROS in mature leaves and could mean that consequently the levels needed to trigger



**Figure 9.** TUNEL Labeling of Control and Myb308 Leaf Sections to Reveal Nuclei Containing Nicked DNA.

(A) to (C) Section of a mature control leaf showing labeling of a single cell in the palisade mesophyll lying just beneath a stoma (arrow in [A]). Labeling of control tissue was infrequent, except in vascular tissue. (B) and (C) show labeling of nuclei in vascular tissue from control leaf sections. Labeled cells were in the differentiating xylem (arrows in [B]).

(D) Labeling of a mature Myb308 leaf section. The majority of nuclei of mesophyll cells stained, as did those of some epidermal cells. Cells with stained nuclei are lying adjacent to dead cells, which did not stain because their cytoplasmic contents are collapsed. Arrows indicate labeling in nuclei of vascular tissue.

(E) Close-up of leaf section from a Myb308 plant showing the labeling of nuclei in the developing xylem (arrows) and the extensive abnormal labeling of mesophyll nuclei. Cells with labeled nuclei are relatively intact but lie adjacent to other dense, collapsed cells within the mesophyll.

**Table 5.** Malondialdehyde Levels in Fully Expanded Leaves of Myb308 and Control Plants

Sample	Malondialdehyde (nmol per mg of Protein) <sup>a</sup>			
	Experiment 1	<i>n</i> <sup>b</sup>	Experiment 2	<i>n</i>
Control	1.94 ± 0.15	3	1.49 ± 0.28	3
Myb308	4.60 ± 1.40	3	2.68 ± 0.31	3

<sup>a</sup> Mean values ± SD are shown.

<sup>b</sup> *n*, number of independent assays.

the HR are reached earlier during the second phase of the oxidative burst. The data do not necessarily imply, however, that the cell death observed in mature Myb308 leaves arises by the same mechanism as that observed in the HR after avirulent pathogen challenge. The cell death phenotype exhibited by Myb308 plants is very similar in many respects to disease lesion mimic mutants in Arabidopsis, maize, and barley (Greenberg and Ausubel, 1993; Wolter et al., 1993; Jabs et al., 1996; Gray et al., 1997). However, mutations affecting a range of cellular functions independent of the HR could give rise to the type of cell death observed in lesion mimic mutants (Greenberg and Ausubel, 1993; Lamb and Dixon, 1997). Such functions could include those that normally operate to contain oxidative damage, as proposed here for phenolic acid derivatives (Greenberg and Ausubel, 1993).

Because overexpression of *AmMYB308* results in a general repression of phenolic acid and monolignol production, it is not possible to identify unambiguously the phenolic acid or derivative that may be primarily responsible for buffering the damaging effects of ROS in tobacco. However, given that this function would be related to the cellular levels of the phenolic, it is likely that chlorogenic acid plays a prominent role, because it is the most abundant phenolic acid derivative in tobacco leaves. Interestingly, in maize, the *Lis1* gene (which suppresses cell death) has been shown to encode a protein with structural similarities to dioxygenases that metabolize phenolics in bacteria (Gray et al., 1997). In this case, the product of the *Lis1* gene has been interpreted as being likely to remove a phenolic mediator of cell death. However, it is also possible that *Lis1* mutant plants fail to synthesize a phenolic derivative important in buffering ROS and tempering cell death, as is the case for Myb308 tobacco. Alternatively, phenolics may play both specific and general roles in modulating plant cell death.

In Myb308 tobacco plants, phenolic derivatives of hydroxycinnamic acids and monolignols play a significant role in the development of leaf palisade cells in tobacco, probably working through the production of phenolic signaling molecules, such as DCGs, that can modify cell division and cell expansion. Phenolic acid metabolism also appears to provide compounds important in controlling the processes of senescence through their ability to buffer cells against the catalytic damage invoked by ROS. Therefore, they have a

significant biological function as natural antioxidants. The analysis of the Myb308 phenotype demonstrates multifunctional roles for phenolic acid/monolignol biosynthesis in plants.

## METHODS

### Plant Material

Control plants were untransformed *Nicotiana tabacum* cv Samsun NN or this cultivar transformed with the binary vector pBin19 to confer kanamycin resistance. Initial phenotypic characterization, transmission electron microscopy, and gravimetric analysis were conducted with four independent primary transformants expressing *AmMYB308* under the control of the cauliflower mosaic virus 35S promoter (Tamagnone et al., 1998). Subsequently, Myb308 plants were selected from the progeny of a single primary transformant overexpressing *AmMYB308*. Plants were grown in the greenhouse at ~22°C with 16-hr days, unless otherwise described.

### Ultrastructural Analysis

For the simple ultrastructural observations, conventional transmission electron microscopy was performed (Stratford et al., 1988). Young leaves, fully expanded (intermediate) leaves, and old leaves from control and Myb308 plants were compared. Leaf samples (~1 cm<sup>2</sup>) were cut and immediately placed in the fixative solution (2.5% glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.2). Samples were cut into small strips (~1 × 2 mm) and vacuum infiltrated with fixative. After replacing the fixative, we left the samples to fix overnight. Postfixation was done in 1% osmium tetroxide for 2 hr.



**Figure 10.** Effect of *AmMYB308* on the Development of the HR to *P. s. maculicola* M2 in Tobacco.

The progress of the HR was monitored by the increase in electrolyte leakage (conductivity) from leaf samples with time after inoculation with the pathogen or water. The onset of the HR was always more rapid in Myb308 leaves than in the controls. The data shown are the results from a representative experiment.



**Figure 11.** Effect of NahG on Cell Death Phenotype Induced by AmMYB308.

A plant homozygous for an insertion constitutively expressing the *nahG* gene to produce NahG (leaf 2) was crossed to a line homozygous for the *AmMYB308* insertion (leaf 1). The phenotypes of leaves from two progeny plants derived from reciprocal crosses are shown (leaves 3 and 4). Clearly, expression of *nahG* does not prevent the premature cell death invoked by AmMYB308, implying that salicylate is not involved in the induction of cell death by AmMYB308.

The strips were then infiltrated in medium grade London Resin white (London Resin Company, Basingstoke, UK) and transferred to gelatine capsules, and the resin was polymerized for 12 hr in a 60°C oven. The embedded samples were sectioned with a Reichert-Jung ultramicrotome (Leica, Milton Keynes, UK) at a thickness of 70 to 90 nm, and the sections were poststained with uranyl acetate and lead citrate (Millonig, 1961). Finally, the specimens were observed using an electron microscope (model 1200 EX; JEOL, Welwyn Garden City, UK).

#### In Situ Detection of Apoptotic Cells

Leaf samples were cut from control and fully expanded Myb308 leaves. Samples were taken from green areas in the middle part of the leaf. Sample fixation and wax embedding were performed in the same way as for in situ hybridization (Jackson, 1992). Nuclei of cells undergoing programmed cell death were stained by the TUNEL method (Gavrieli et al., 1992) by using the ApopTag in situ apoptosis detection kit (peroxidase version) (Appligene; Oncor, Birtley, UK). The method was adapted from that recommended by the manufacturer. Wax-embedded samples were sectioned to a thickness of 7  $\mu\text{m}$  and then dewaxed in xylene (two washes of 5 min each), absolute ethanol (two washes of 5 min each), 95% ethanol and then 70% ethanol for 3 min each wash, and finally PBS (10 mM phosphate buffer and 145 mM NaCl, pH 7.2) for a 5-min wash. Proteins were digested with proteinase K (20  $\mu\text{g}/\text{mL}$ ) and then washed four times in distilled water for 2 min for each wash. Endogenous peroxidase activity was quenched by treating the slides in 1% hydrogen peroxide in PBS for 5 min at room temperature. Slides were then rinsed twice with PBS for 5 min each, and then terminal transferase equilibration buffer (Appligene;

Oncor) was added. Terminal transferase and dUTP-digoxigenin were then added to the reaction buffer, as recommended by the manufacturer (Oncor). The reaction ran at 37°C for 30 min. After washing three times in PBS, the anti-digoxigenin-peroxidase antibody conjugate was applied to the slides and incubated for 30 min at room temperature. The specimens were then washed three times in PBS and once in distilled water. The peroxidase substrate, consisting of 4.8 mL of distilled water, 1.2 mL of chloronaphthol (30 mg/mL in methanol), and 7.5  $\mu\text{L}$  of  $\text{H}_2\text{O}_2$ , was then added. The substrate was allowed to react for a few minutes, and the slides were then washed copiously with distilled water. Sections were mounted in water and photographed immediately by using bright-field microscopy. Controls in which water was used in place of terminal transferase were included and showed no labeling of nuclei.

#### Malondialdehyde Assay

Fresh leaves (0.5 g) were ground in 3 mL of 10 mM sodium phosphate buffer, pH 7.2, and then centrifuged at 2000g for 10 min. A sample of the supernatant (0.2 mL) was added to a glass tube containing 0.8 mL of distilled water, 0.5 mL of 20% (w/w) trichloroacetic acid, and 1 mL of 10 mM thiobarbituric acid. A control was run for each sample in which thiobarbituric acid was replaced by an equal volume of distilled water. The samples were heated in a boiling water bath for 30 min and then centrifuged for 10 min at 2000g to remove haziness. The cleared samples were allowed to equilibrate to room temperature before the absorption at 532 nm was measured. The concentration of malondialdehyde (MDA) was calculated using its extinction coefficient of 156  $\text{mmol cm}^{-1}$ .

#### NahG Plants

A homozygous line of tobacco expressing the *nahG* gene from *Pseudomonas putida* under the control of the cauliflower mosaic virus 35S promoter was provided by J. Draper (Bi et al., 1995). This line was crossed reciprocally to a line homozygous for *AmMYB308*. Progeny from both crosses showed 100% resistance to kanamycin and developed lesions on their leaves.

#### Gravimetric Analysis

Before harvesting leaves, we kept plants in complete darkness for 2 days to allow starch to mobilize; otherwise, the starch would sediment with the cell wall pellets. This period of darkness was sufficient to obtain cell wall pellets free of starch, as determined by microscopic observation and iodine staining.

Fully expanded leaves of three control lines and Myb308 lines derived from four independent primary transformants were halved by cutting along their midveins. One-half of each leaf was weighed, freeze-dried, and weighed again to give the total dry weight. The other half of each leaf was weighed and used for preparing crude cell walls. Leaf material was frozen in liquid nitrogen and ground to a fine powder. The powder was resuspended in 40 mL of distilled water and then centrifuged at 50g for 5 min at 4°C to obtain a pellet comprised mainly of cell walls. The supernatant was removed, and the pellet was washed twice in 80% ethanol and once in distilled water. Samples were then freeze-dried and weighed. The dry weight of the

freeze-dried cell wall material was determined. Supernatants from all the washes during the cell wall preparation were pooled and freeze-dried. This dry weight was considered the soluble fraction.

#### Measurement of Cell Sizes

Measurements of the long axis, short axis, maximum perimeter, and maximum section area of cells were taken using a computerized graphic tablet from scanning electron micrographs of 10 control and 10 Myb308 leaf blades. The figures obtained were used to calculate the average surface area and volume of palisade cells in leaves of different ages. The mathematical formulas used were from Korn and Korn (1968).

#### Establishment of Cell Suspension Cultures

Seeds of control and Myb308 plants were surface-sterilized and sown individually in 100-mL Richardson's (Leicester, UK) jars with 20 mL of Murashige and Skoog (MS) plant salt and vitamin mixture (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.8% Difco bacto agar. Seeds were germinated and grown aseptically for 4 weeks at 25°C with 16-hr days. Cut pieces of small leaves were placed in Petri dishes with 15 mL of MS agar supplemented with 2 mg/L 2,4-D and 100 µg/mL kanamycin and placed in the dark for ~3 weeks until calli were formed. The calli were then transferred into liquid culture by placing them in 250-mL conical flasks containing 50 mL of MS medium, pH 5.8 (Murashige and Skoog, 1962), supplemented with 3% sucrose, 100 mg/L inositol, 1 mg/L thiamine, 0.2 mg/L 2,4-D, and 200 mg/L KH<sub>2</sub>PO<sub>4</sub>. Flasks were placed on an orbital shaker rotating at 100 rpm and grown at 25°C under constant dim light. The cultured cells were subcultured once a week by placing 10 mL of the culture in a flask containing fresh MS medium.

To measure cell culture doubling time, we subcultured 5 mL of each culture into 50 mL of fresh medium. A 5-mL sample of the culture was taken at the start and at various time points during the ensuing 8 days. Cells were counted using a hemocytometer, and the total number of cells in a 5-mL sample was calculated.

#### Production and Feeding of Dehydrodiconiferyl Alcohol

Dehydrodiconiferyl alcohol (DCA) was produced according to Teutonico et al. (1991) by using coniferyl alcohol and horseradish peroxidase (Sigma). DCA was analyzed by HPLC as described by Tamagnone et al. (1998). The feeding experiment was done by subculturing control and Myb308 cells into different flasks containing 50 mL of the medium plus DCA (100 µM), dehydrodiconiferyl alcohol glucoside (DCG; 10 µM), or zeatin riboside (0.1 µM). Samples (0.5 mL) were taken daily for microscopic observation.

#### Extraction and Measurement of Phenolics

Plantlets grown in culture, maturing leaves, or suspension cultured cells were frozen in liquid nitrogen and ground with a pestle and mortar. Phenolics were extracted in dry, ice-cold methanol and analyzed by HPLC, as described by Tamagnone et al. (1998). For identification

of DCA and isomers of DCG, samples were compared with standards prepared as described by Lynn et al. (1987) and verified by nuclear magnetic resonance and mass spectrometry. Positive identification of DCA and DCG isomers involved coelution with the standards and demonstration of the appropriate, characteristic UV absorption spectra profiles.

#### Inoculation of Plants with *Pseudomonas syringae*

*P. syringae* pv *maculicola* M2 was prepared as a bacterial suspension at 10<sup>8</sup> colony-forming units per milliliter of water and used to flood large sectors of leaf mesophyll of control and Myb308 plants grown in growth cabinets at 22°C under constant illumination. Development of the hypersensitive response (HR) was monitored visually. Two leaf discs from each inoculated area were placed in 10 mL of high-purity deionized water of known conductivity. The discs were vacuum infiltrated for 2 min and then left to stand for 30 min at 22°C. The conductivity of the water was then measured.

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