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RESEARCH ARTICLE

The AmMYB308 and AmMYB330 Transcription Factors from Antirrhinum Regulate Phenylpropanoid and Lignin Biosynthesis in Transgenic Tobacco

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MYB-related transcription factors are known to regulate different branches of flavonoid metabolism in plants and are believed to play wider roles in the regulation of phenylpropanoid metabolism in general. Here, we demonstrate that overexpression of two *MYB* genes from Antirrhinum represses phenolic acid metabolism and lignin biosynthesis in transgenic tobacco plants. The inhibition of this branch of phenylpropanoid metabolism appears to be specific to AmMYB308 and AmMYB330, suggesting that they recognize their normal target genes in these transgenic plants. Experiments with yeast indicate that AmMYB308 can act as a very weak transcriptional activator so that overexpression may competitively inhibit the activity of stronger activators recognizing the same target motifs. The effects of the transcription factors on inhibition of phenolic acid metabolism resulted in complex modifications of the growth and development of the transgenic plants. The inhibition of monolignol production resulted in plants with at least 17% less lignin in their vascular tissue. This reduction is of importance when designing strategies for the genetic modification of woody crops.

INTRODUCTION

Several of the genes involved in the control of flavonoid biosynthesis in plants have been shown to encode transcription factors structurally related to the c-MYB protooncogene family of mammals (Cone et al., 1986, 1993a, 1993b; Paz-Ares et al., 1986, 1987; Quattrocchio et al., 1993; Grotewold et al., 1994; Quattrocchio, 1994). The family of genes encoding MYB-related transcription factors in higher plants is large (Martin and Paz-Ares, 1997), and the functions of the majority of members are not known. However, biochemical studies have suggested that the involvement of MYB-related transcription factors may extend beyond the control of flavonoid metabolism and include other branches of phenylpropanoid metabolism. For example, the AmMYB305 and AmMYB340 proteins that were first characterized in Antirrhinum flowers (Jackson et al., 1991) are able to transactivate genes encoding phenylalanine ammonia–lyase (PAL) in tobacco and appear to control the activation of this primary step of phenylpropanoid metabolism in flowers of Antirrhinum as well as later steps involved in flavonol metabolism (Sablowski et al., 1994, 1995; Moyano et al., 1996).

Many of the genes encoding the enzymes of general phenylpropanoid metabolism, such as PAL, cinnamate 4-hydroxylase (C4H), and 4-coumaroyl–CoA ligase (4CL), and the enzymes involved in synthesis of lignin precursors, such as caffeic acid *O*-methyltransferase (COMT) and cinnamyl alcohol dehyrogenase (CAD), are shown in Figure 1. They contain motifs conserved within their promoters that conform well to the motifs recognized by plant MYB transcription factors (Bugos et al., 1991; Hauffe et al., 1993; Shufflebottom et al., 1993; Sablowski et al., 1994; Ye et al., 1994; Feuillet et al., 1995; Solano et al., 1995; Douglas, 1996), and in a number of

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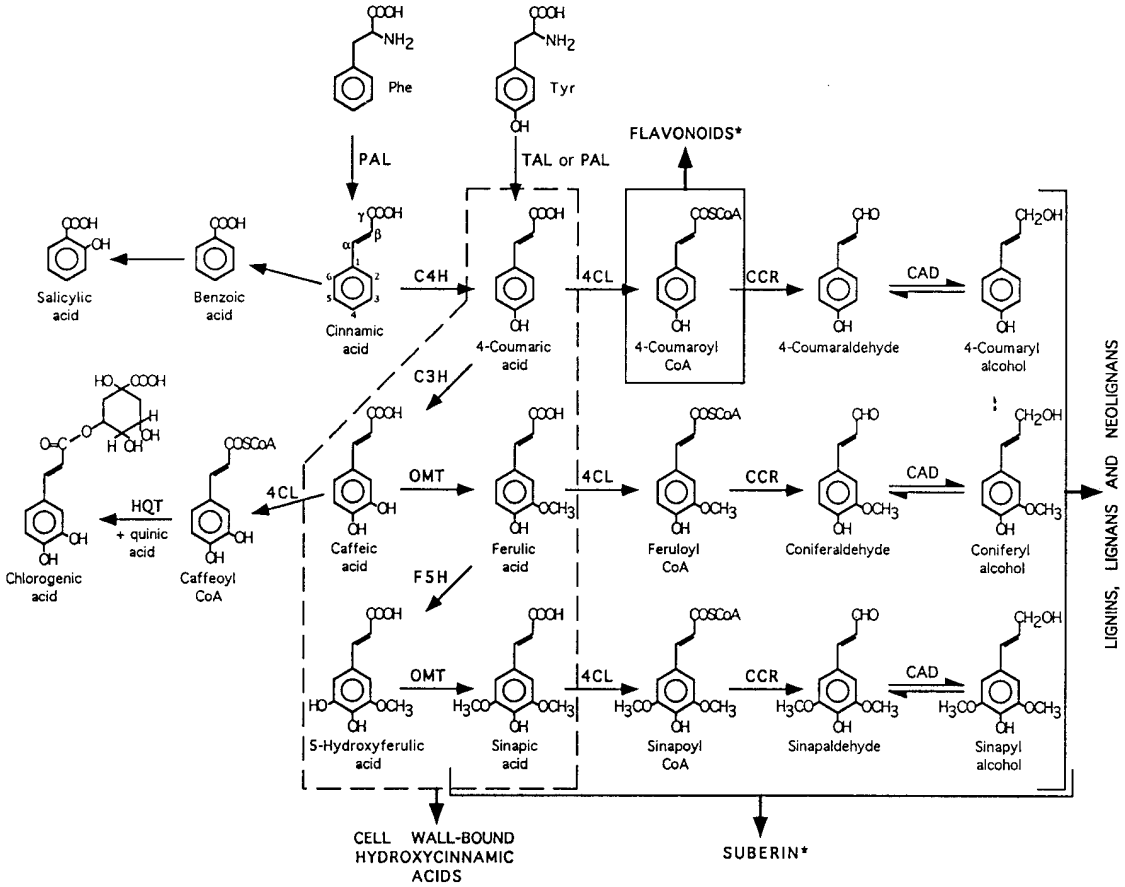

Figure 1. Pathway Involved in the Synthesis of Monolignols and Phenolic Acids (Hydroxycinnamic Acids) and Their Derivatives.

The asterisk indicates that the polyketide (acetate) pathway is also required for both flavonoids and suberin synthesis. CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl–CoA reductase; C3H, 4 coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl–CoA ligase; F5H, ferulate 5-hydroxylase; HQT, hydroxycinnamoyl–CoA quinate transferase; OMT, *O*-methyltransferase; PAL, phenylalanine ammonia–lyase; TAL, tyrosine ammonia–lyase.

cases, these motifs have been demonstrated to be involved functionally in the control of phenylpropanoid gene expression (Hauffe et al., 1993; Sablowski et al., 1994).

As part of a program characterizing MYB transcription factors in plants, we originally isolated six *MYB* genes that were expressed highly in the flowers of Antirrhinum (Jackson et al., 1991). The deduced amino acid sequences of two of them, AmMYB308 and AmMYB330, are particularly similar in their DNA binding domains (94% identical), suggesting that they may bind the same target DNA motifs. These two proteins show no conservation of sequence in their C termini; however, AmMYB308 contains motifs found in MYB transcription factors isolated from other plant species, such as maize and barley (Marocco et al., 1989; Jackson et al., 1991). These structural features suggest that AmMYB308 and AmMYB330 may recognize the same or very similar target motifs; however, because of the divergence in their C termini, they may have distinct functions in regulating transcription. *AmMYB308* is expressed throughout Antirrhinum plants, whereas *AmMYB330* is expressed primarily in mature flowers.

To study the functions of *AmMYB308* and *AmMYB330*, we made constructs of both genes in the sense and antisense orientations driven by the strong cauliflower mosaic virus (CaMV) 35S promoter and used them to transform tobacco. Plants overexpressing *AmMYB308* and *AmMYB330* in the sense orientation showed a phenotype that differed in many respects from that of the wild-type plants. These transgenic plants therefore provided us with an opportunity to investigate the functions of these two genes. In this study, we report results indicating a role for both genes in the regulation of phenylpropanoid and lignin biosynthesis.

RESULTS

Phenotypes of Tobacco Plants Overexpressing *AmMYB308*

Twenty-one independent primary transformants overexpressing *AmMYB308* under the control of the double CaMV 35S promoter (Figure 2B) were examined. Ten had an altered phenotype (Myb308 phenotype) when compared with control plants (i.e., wild-type plants or plants transformed with the binary vector pBIN19 alone). The mature Myb308 plants were stunted and never grew as quickly as the controls did (Figure 3A), due partly to reduced elongation of the stem internodes during development. Other effects of overexpressing *AmMYB308* were particularly apparent on the leaves themselves (Figures 3A, 3B, and 3D). In young leaves, the photosynthetic tissue was paler than in control leaves, although the veins and the tissues immediately adjacent to them remained darker green, making the vascular network easily visible (Figure 3B). The size of the leaves in Myb308 and control plants was similar during the early stages of development but started to differ as the leaves began to expand rapidly, so the mature leaves of Myb308 plants were much smaller than those of the control plants.

The most striking characteristic of the leaves of the Myb308 plants was the appearance of isolated patches of dead cells from the time that the leaves reached maturity. Patches of dying cells arose initially in the tip region of the leaf and subsequently spread along the edges and inward to affect all parts of the leaf, except the midvein and a few of the major veins (Figures 3A and 3D). Finally, as the leaves aged, cell death spread to all tissues of the leaf, including the veins. The areas of dead tissue were white in appearance, in contrast to the brown color of old, senescent wild-type leaves or of young leaf tissue from tobacco plants after injury (which assumes a brown color as it dies). The areas of white, dead tissue appeared on the leaves of Myb308 plants earlier than did the brown color associated with senescence of control leaves.

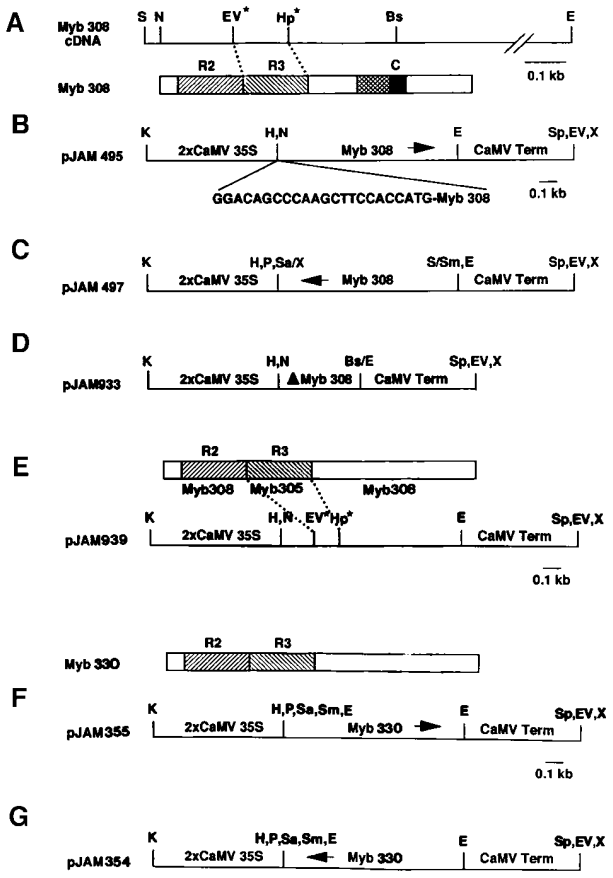

Figure 2. Restriction Maps of the *AmMYB308* and *AmMYB330* Gene Constructs Used to Transform Tobacco.

(A) The *AmMYB308* cDNA with the NcoI site introduced over the first ATG of the coding sequence. The regions of protein encoded by the *AmMYB308* cDNA are illustrated below the cDNA restriction map, R2 and R3 being the two repeats of the MYB DNA binding domains equivalent to repeats 2 and 3 in c-MYB. The region of potential amphipathic α-helix is crosshatched, and the conserved motif found in some other plant MYB proteins (Marocco et al., 1989) is shown in black in rectangle C. The asterisks mark HpaI and EcoRV restriction sites introduced for the exchange of the region encoding R3 in the construction of pJAM939.

(B) The gene construct pJAM495 that placed the *AmMYB308* cDNA under the control of the CaMV 35S promoter. The sequence at the junction between the double CaMV 35S promoter (2 $\times$ CaMV 35S) and the *AmMYB308* cDNA is shown in detail to illustrate the Kozak consensus initiation site. The arrow indicates the orientation of the *AmMYB308* coding sequence.

(C) The *AmMYB308* antisense construct pJAM497.

(D) pJAM933, which removed part of the *AmMYB308* sequence encoding the C terminus of the AmMYB308 protein, including part of the conserved motif C.

(E) Chimeric *AmMYB308*/*AmMYB305* gene. The third repeat of AmMYB305 (R3) was used to replace the R3 of AmMYB308 by engineering EcoRV and HpaI sites (asterisks) at the beginning and at the end of the DNA sequence encoding this region in each cDNA clone. The resultant gene construct driven by the CaMV 35S promoter was pJAM939.

(F) The gene construct pJAM355 that placed the *AmMYB330* cDNA under control of the CaMV 35S promoter. The arrow indicates the orientation of the *AmMYB330* coding sequence.

(G) The corresponding *AmMYB330* antisense gene construct pJAM354.

Bs, BsaBI; Bs/E, fusion of BsaBI and EcoRI sites; E, EcoRI; EV, EcoRV; H, HindIII; Hp, HpaI; K, KpnI; N, NcoI; P, PstI; S, SspI; Sa, SalI; Sa/X, fusion of SalI and XhoI sites; Sm, SmaI; S/Sm, fusion of SspI and SmaI sites; Sp, SphI; Term, terminator; X, XhoI.

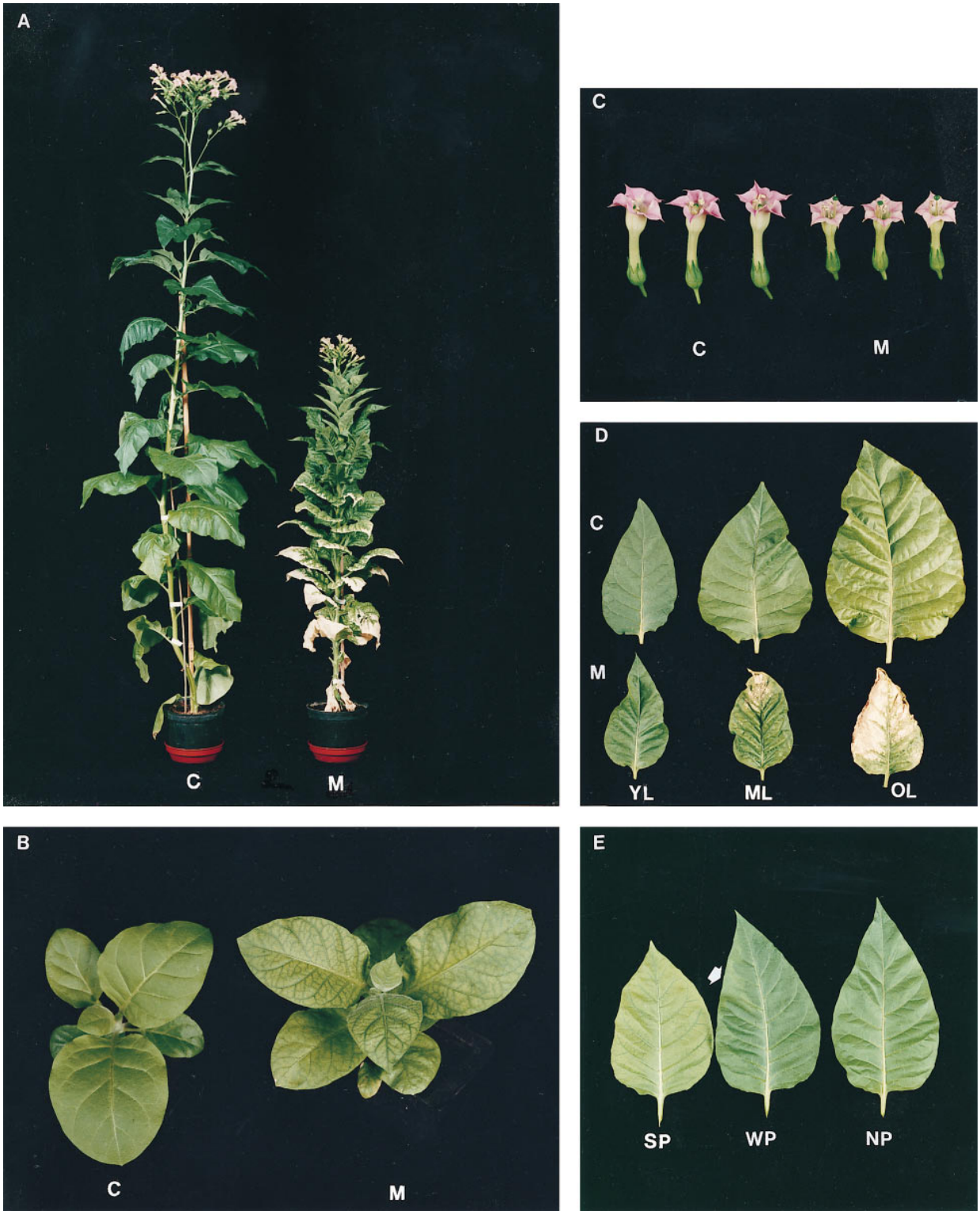

Figure 3. Phenotypic Changes Induced by the Overexpression of *AmMYB308* in Tobacco.

The morphology of Myb308 flowers was also affected (Figure 3C). The corollas were reduced in size and were less pigmented, particularly in the areas between each petal midvein, so the flowers appeared patterned. The stigma grew to a normal size and protruded above the corolla; the stamens also protruded from the corolla; however, unlike control plants, they were significantly shorter than the stigma, making self-pollination infrequent (Figure 3C). However, hand pollination resulted in the production of viable seed.

Phenotype of Myb330 Plants

Expression of *AmMYB330* under the control of the double CaMV 35S promoter gave a phenotype very similar to that conferred by *AmMYB308* (Figure 4). Ten plants from a total of 35 independent transformants showed reduced growth rates and paler leaves. In mature leaves, white lesions were also observed. Although the phenotype of leaves with high expression of either *AmMYB330* or *AmMYB308* was essentially identical, in the flowers of Myb330 plants, the petals were not significantly smaller. In addition, when compared with the untransformed control plants, there was not conspicuously less anthocyanin present.

Segregation of the Myb308 Phenotype

Five of the primary transformants that overexpressed *AmMYB308* and showed the Myb308 phenotype were self-fertilized, and their seeds were sown on agar containing kanamycin (100 μg mL^{-1}). The phenotype caused by overexpression of *AmMYB308* was visible at the seedling stage; the cotyledons were slightly larger and more pointed than normal, and their color was paler, except along the veins. Small lesions occasionally developed spontaneously on these cotyledons, even under sterile conditions.

Seedlings of the line of tobacco used for transformation (Samsun NN) do not bleach when grown on kanamycin. They can be identified as sensitive to kanamycin because they do not develop true leaves, and their root systems remain small. Because the Myb308 phenotype could also be scored on the cotyledons, the progeny of the primary transformants could be scored both for resistance to kanamycin and for the Myb308 phenotype. The two traits always segregated together. In the progeny of one plant, the ratio was 3:1 (kanamycin resistant/Myb308 to kanamycin sensitive), indicating a single insertion governing both dominant traits; in the other plants, the scores indicated a ratio of 15:1, corresponding to two unlinked insertions conferring both the resistance to kanamycin and the Myb308 phenotype. In the progeny of three independent transformants expressing *AmMYB330* (and showing a phenotype similar to that caused by *AmMYB308*), kanamycin resistance also cosegregated consistently with the altered phenotype.

Some of the progeny of the primary Myb308 transformants were grown to maturity. In one family, the majority of kanamycin-resistant progeny (64%) retained a strong phenotype with all of the characteristics described above; 36% had a weaker phenotype, with growth rates and leaf sizes close to those of the control. However, leaves of plants with the weaker phenotype developed extensive lesions as they approached maturity (Figure 3E). These findings indicated different degrees of penetrance of the Myb308 phenotype in this family. Gel blotting of DNA from these plants showed two unlinked T-DNA insertions. One of these was always present in plants showing the strong phenotype (data not shown), suggesting that the contribution of one insertion to the Myb308 phenotype was greater than the contribution of the other insertion.

Gel blot analysis of RNA from leaves of individual progeny plants within this family (including those shown in Figure 3E) showed that the level of expression of *AmMYB308* correlated well with the penetrance of the phenotype (Figures 5A and 5B). Relatively high expression was found in plants with the strong phenotype, and relatively low expression was observed in plants with the weaker phenotype. To confirm that the RNA samples were comparable, RNA gel blots were also

Figure 3. (continued).

(A) Wild-type (C) and Myb308 (M) tobacco plants at the flowering stage. Note the stunted posture and the consequent crowding of the leaves in the transgenic plant. Note also the white lesions that are starting to appear on the leaves as they approach full expansion; eventually, the white lesions cover the entire blade in older leaves.

(B) Young control (C) and Myb308 (M) plants seen from above. Note the pale photosynthetic tissue between the veins in the leaves of the transgenic plant. The vascular system stands out as a darker green network.

(C) Flowers of control (C) and Myb308 (M) plants. Note the smaller size of the corolla and sepals in the transgenic flowers. This allows the stigma and stamens to protrude from the corolla; however, the stamens are shorter than the stigma, thereby reducing self-fertilization. Note also the pigment deficiency in the corollas of Myb308 flowers.

(D) Young leaves (YL), mature leaves (ML), and old leaves (OL) in control (C) and Myb308 (M) plants. Note the failure of Myb308 leaves to expand to full size and the spreading lesions in the leaf blade.

(E) Strong phenotype (SP) and weak phenotype (WP) in leaves from corresponding Myb308 transgenic plants, segregating in the progeny of one primary transformant. A leaf from a control plant not expressing AmMYB308 and showing no phenotype (NP) is shown for comparison. The arrow indicates a patch of leaf tissue showing a stronger Myb308 phenotype in a leaf that generally showed only a weak phenotype.

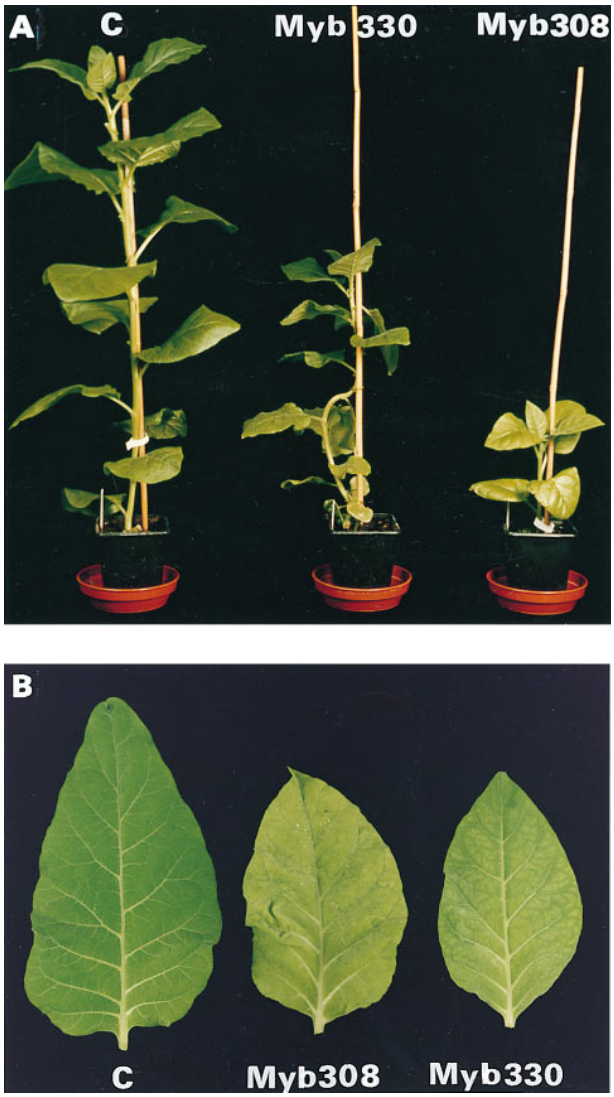

Figure 4. Phenotypic Changes Induced by Overexpression of *AmMYB330* in Tobacco.

(A) Untransformed control (C), a primary transformant expressing *AmMYB330* (Myb330), and a plant homozygous for the *AmMYB308* transgene (Myb308). Control and Myb330 plants were the same age and were grown under the same conditions. The Myb330 phenotype resembles that of the Myb308 phenotype in that plants grow slowly, leaves are pale, and mature leaves have white lesions.

(B) Leaves from an untransformed control (C), a primary transformant expressing *AmMYB330* (Myb330), and a plant homozygous for the *AmMYB308* transgene (Myb308).

hybridized with a probe encoding the small subunit of ribulose bisphosphate carboxylase (*RBCS*) from tobacco and with a cDNA clone from Antirrhinum encoding PAL (Martin et al., 1991). Both the *RBCS* and *PAL* transcript levels were equal in all Myb308 plants and in control plants (Figures 5A and 5B). Therefore, we conclude that the strength of penetrance of the Myb308 phenotype in the transgenic plants depended on the relative levels of expression of the introduced *AmMYB308* gene.

The expression of *AmMYB330* in three independent transgenic lines showing a modified phenotype was compared with the expression in independent lines showing normal phenotypes. The modified phenotype, including paler leaves and white lesions, was also found exclusively in those lines expressing high levels of *AmMYB330* (data not shown).

Biochemical Basis for the Myb308 and Myb330 Phenotypes

One of the most striking features of the Myb308 phenotype was the appearance of the mature leaves, which were white as the tissue died rather than brown, as occurred in senescent leaves of the controls (Figure 6A). This suggested that phenolics, normally present in tobacco leaves, were not being oxidized in the aging and dying tissue to give the usual brown color. This could be due either to an absence of phenolics or to a lack of the enzymes that normally catalyze their oxidation. Extracts of control and transgenic plants were made in phosphate buffer and separated into soluble fractions and insoluble cell wall fractions by centrifugation. The insoluble fractions were cleaned by washing copiously with deionized water. Both the soluble fractions and the cell wall fractions from control plants were tinted brown relative to extracts from Myb308 plants (Figure 6B). HPLC analysis of the soluble extracts from control tissue indicated that the brown coloration was due to a mixture of oxidized phenolics (data not shown). However, enzyme assays showed that peroxidase and laccase activities were comparable in control and Myb308 plant extracts (data not shown).

Analysis of Phenolics in Leaves

To determine whether the transgenic plants had reduced levels of soluble phenolics, we used ice-cold methanol to extract leaves from transgenic plants that were transformed either with *AmMYB308* or with *AmMYB330*, and then we analyzed them by HPLC. There were striking differences between samples of whole leaves from Myb308 and control plants. The greatest variety of phenolics was found in young leaves of the wild-type plants, with 17 major peaks being recognized (Figure 7A). Comparison to standard spectra showed that most of these peaks represented soluble esters of caffeic and ferulic acid, with the most abundant being caffeoyl quinate (chlorogenic acid). Extracts from Myb308 leaves were found to be deficient in all of these soluble esters, with reductions ranging between 70 and 98% (Figures 7B and 7D). The most abundant phenolic was still chlorogenic acid, but it was reduced, on average, by 80% (com-

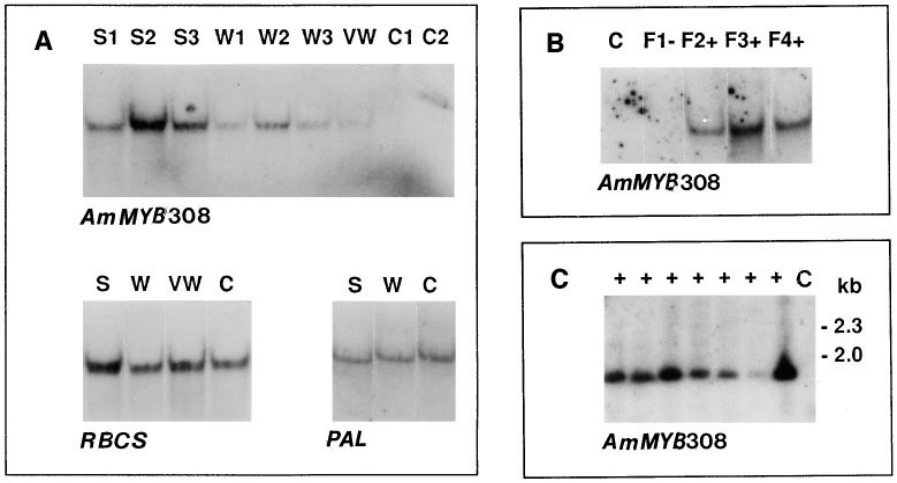

Figure 5. Expression of *AmMYB308* in the Transgenic Plants.

(A) Correlation of *AmMYB308* expression to the penetrance of the Myb308 phenotype observed in the progeny of one primary transformant. Total RNA (15 μg) from leaves 5 to 9 of three plants showing the strong phenotype (S1, S2, and S3), from three leaves showing the weak phenotype (W1, W2, and W3), from a single plant showing a very weak phenotype (VW), and from controls of either untransformed Samsun (C1) or a plant transformed with another *MYB* gene (*AmMYB315*) construct (C2) was separated on RNA gel and probed with the *AmMYB308* cDNA. RNA samples from these plants were also probed with a clone encoding the small subunit of ribulose bisphosphate carboxylase (*RBCS*) from tobacco and one encoding PAL from Antirrhinum. The expression of both *RBCS* and *PAL* was the same in plants showing different degrees of penetrance of the Myb308 phenotype and in controls.

(B) RNA gel blots from plants derived from seed from four different primary transformants (F1, F2, F3, and F4) which either showed (+) or did not show (−) the Myb308 phenotype compared with RNA from untransformed Samsun leaves (C).

(C) Gel blot of genomic DNA (10 μg) from plants showing the Myb308 phenotype (+) cut with EcoRI, which should release a fragment of 1.9 kb from the pJAM495 construct to be detected with a probe from the *AmMYB308* cDNA sequence. No hybridization was detected in DNA from untransformed Samsun NN plants (C). Numbers at right indicate the lengths of marker DNA fragments in kilobases.

pare peak 7 in Figures 7A and 7C with peak 7 in Figures 7B and 7D). Two peaks (13 and 15) representing flavonoids were less affected and showed reductions of ~50%. In contrast, a tryptophan derivative eluting as peak 10 was slightly increased in the extracts from young leaves of Myb308 plants. These differences in phenolics were observed in extracts from all stages of leaf development but were particularly striking in older leaves of Myb308 plants (Figure 7D) in which all soluble phenylpropanoid derivatives were virtually absent, including the two flavonoid peaks. Only the tryptophan derivative appeared to be relatively unaffected. HPLC analysis of extracts from other parts of the plants (stems and flowers) and from whole seedlings revealed a similar pattern of reduction of all major phenolics; however, the flavonoids in flowers were less severely reduced (results not shown). The reproducibility of the results was good when plants with strong phenotypes were used; in one experiment in which a plant with a weaker phenotype was used for analysis, the reduction in phenolics was less marked but still conspicuous. We also extracted phenolics from cell wall preparations and found a general reduction in a wide range of phenolic derivatives, with some derivatives more reduced than others (Figures 7E and 7F).

Analysis of soluble phenolics from two Myb330 plants with a strong phenotype showed that a significant reduction in all soluble phenolic compounds in the leaves was associated with the expression of *AmMYB330.* Two other plants derived from the transformation experiment with the *AmMYB330* gene construct were also used in this analysis. They did not have an altered phenotype and did not express *AmMYB330.* They showed wild-type levels of soluble phenolics. The differences in chlorogenic acid content of plants expressing and not expressing *AmMYB330* and comparable measurements from controls and Myb308 plants are given in Table 1.

Additional evidence for a deficiency in phenolics in Myb308 plants came from the observation that Myb308 leaves viewed under long-wave UV light fluoresced red-orange (Figures 6C and 6D). We concluded that the Myb308 leaves lack phenolic compounds that normally absorb UV light. In their absence, UV light can excite the chlorophyll in mesophyll cells, causing them to fluoresce red-orange in the same way as reported for the sin1 mutant of Arabidopsis by Chapple et al. (1992). The red-orange appearance was reproducibly obvious under UV light in leaves from Myb308 plants with strong phenotypes of all ages, indicating a

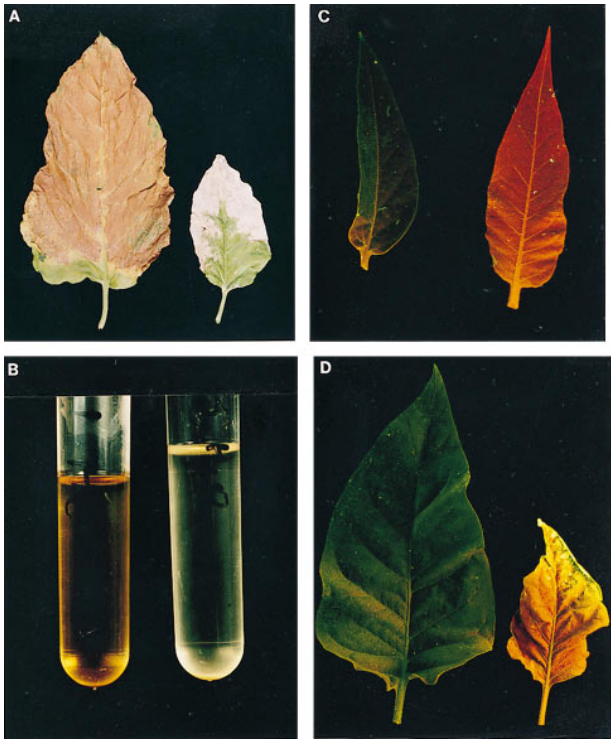

Figure 6. Visible Signs of the Absence of Phenolic Compounds in Myb308 Plants.

(A) Comparison between a senescent leaf from an old control plant (left) and a Myb308 leaf (right). Note the brown coloration of the control leaf as opposed to the whiteness of the dead tissue on the Myb308 leaf. The brown coloration is given by the presence of large amounts of oxidized phenolics, which are absent in the Myb308 leaf.
(B) Soluble extracts of leaf tissues from control (left) and Myb308 (right) leaf samples. The supernatants were prepared by grinding frozen leaf tissue in water. They were clarified by centrifugation followed by filtration through a 10-kD Amicon filter. Note the brown coloration of the control filtrate as opposed to the translucent pale yellow color of the Myb308 filtrate. These results were reproducibly obtained from four independent Myb308 lines and three Myb330 lines.
(C) Appearance of control (left) and Myb308 (right) young leaves photographed under UV light (365 nm). Note the red-orange appearance of the Myb308 leaf as opposed to the dark green of the control leaf.
(D) As shown in **(C)**, but using mature leaves as samples. The Myb308 leaf (right) appears red-orange in the areas where chlorophyll is present and yellow-white in the areas of dead tissue. The control leaf (left) appears dark green.

general reduction in phenolics within the entire plant. However, red-orange coloration was less evident in plants with the weaker phenotype. In contrast, control plants appeared dark blue-green in color under the same conditions. Overexpression of *AmMYB330* resulted in a phenotype similar to that conferred by *AmMYB308* in transgenic plants and similar significant reductions in phenolic levels in leaves.

These results imply that both transcription factors repress the branch of phenylpropanoid metabolism that leads to the synthesis of phenolics, such as chlorogenic acid, and related compounds, such as ferulic acid, located in cell walls. This biosynthetic pathway is relatively well characterized (Figure 1).

To assess the molecular effects of AmMYB308 and AmMYB330 on phenylpropanoid metabolism in transgenic tobacco, we isolated cDNA clones encoding some of these structural genes from tobacco and compared the steady state levels of transcripts of these genes in wild-type and transgenic leaves. The expression of PAL was unaffected by AmMYB308, confirming our previous observations in the analysis of the transgenic phenotype. However, the expression of genes encoding C4H, 4CL, and CAD was significantly and reproducibly reduced in Myb308 plants (Figure 8A). The expression levels of all of these genes were very low in normal leaf tissue, implying that the reductions observed in Myb308 plants may be great enough to influence flux along the pathway. We also analyzed the activities of PAL and 4CL enzymes in control and transgenic leaves. The basal level of 4CL activity was reduced (between four- and sevenfold) in Myb308 plants (Figure 8B), whereas the basal level of PAL activity was unaffected, confirming that the reduced levels of transcripts caused reduced activity of the enzymes of phenolic metabolism. However, wounding of the leaf tissue before enzyme assay resulted in the induction of 4CL and PAL activities in both control and transgenic plants, although the maximum levels of 4CL activity were reduced in Myb308 lines compared with the controls (Figure 8B). The fact that the transcription factor has its greatest effect on the basal level activity of 4CL suggests that perhaps additional *4CL* genes are switched on by an independent induction mechanism in response to wounding to fulfill the need for extra phenolic compounds. Alternatively, an independent activator may operate to induce *4CL* gene expression in response to wounding, and it may be able to override the repressing effects of AmMYB308 (such possibilities for the control of the *4CL* gene family are discussed in Campbell and Sederoff [1996]).

We compared the reductions in *4CL* gene expression in Myb308 plants to the levels in Myb330 plants and in controls. Transcript levels of the gene encoding 4CL were reduced by a similar degree in Myb330 and Myb308 leaves compared with untransformed controls (Figure 9A). We also analyzed the effect of AmMYB308 and AmMYB330 on flavonoid metabolism by comparing transcript levels of the gene encoding chalcone synthase (CHS) in flowers from control and transgenic plants. There was no significant effect of *AmMYB330* on *CHS* transcript levels in tobacco flowers, although there was a slight (twofold) reduction in *CHS* transcript levels in Myb308 plants (Figure 9B). Although this was relatively small compared with the effects of either AmMYB308 or AmMYB330 on the genes of phenolic metab-

olism, it suggested slight differences in the activity of the two transcription factors in their ability to repress the genes of flavonoid metabolism. Such differences could account for the different effects of overexpression of the two transcription factors on the anthocyanin content of tobacco flowers.

Effect of Expression of the *AmMYB308* Transgene on Lignin Production in Vascular Tissue

Phenylpropanoid precursors are necessary for the biosynthesis of lignin, so lignin formation in stem sections was examined by histological staining. The reaction with phloroglucinol produced a bright red coloration uniformly distributed throughout the thick cell walls of wild-type xylem vessels. In contrast, the xylem vessels of Myb308 plants either did not stain or stained only faintly (Figure 10). Despite this significant quantitative effect on lignin in the vascular tissues, the overall histology of the secondary thickened tissues did not appear to be altered in the transgenic plants; neither was there any red coloration of the unstained xylem, as has been observed in maize lines with mutations of the genes encoding the late steps in lignin biosynthesis, such as COMT (Vignols et al., 1995), and in tobacco with reduced levels of CAD expression (Halpin et al., 1994).

The Mäule reagent stained the xylem vessels uniformly in the stems of both wild-type and Myb308 plants. However, the intensity of the deep red coloration was much greater in the wild-type samples (Figure 10). A very clear-cut difference in staining with the Mäule reagent was also found in leaf traces (not shown) and in the fibers associated with the internal and external phloem tissue, which never stained in the samples from Myb308 plants but which were always stained intensely in samples from the wild type.

Qualitative and Quantitative Effects on Lignin

Nitrobenzene oxidation of lignin released both vanillin and syringaldehyde from all samples; vanillin is indicative of guaiacyl (G) units and syringaldehyde is indicative of syringyl (S) units in lignin derived from coniferyl alcohol and sinapyl alcohol monolignols, respectively (Chen, 1992). The amounts and ratios of the oxidation products were different between wild-type and Myb308 samples (Table 2). In younger parts of Myb308 stems, only the S units were affected by the expression of AmMYB308. This matched well with the observations that the younger lignin of the leaf traces was not stained by the Mäule reagent. In more mature lignin of the older parts of Myb308 stems, the amount of G units was also appreciably reduced, which matched well with the reduced phloroglucinol staining of this lignin (Figure 9). As a consequence, the G/S ratio was inclined much more toward the S units in samples from older stems than in control samples in which there were almost equal proportions of G and S units. Considering the changes in both G and S units together, the reduction in total lignin quantity in Myb308 plants was estimated by this method to be 17.5% in the younger parts of the stem and 56.5% in the mature bottom parts.

Antisense Expression of *MYB308*

In contrast to the strong phenotypes associated with sense overexpression of *AmMYB308* and *AmMYB330*, tobacco plants overexpressing *AmMYB308* or *AmMYB330* in antisense orientation did not show any evident phenotypic changes. This failure to observe a phenotypic change could have been because the Antirrhinum genes were unable to inhibit expression of the orthologous tobacco genes through antisense due to limited sequence homology.

Molecular Basis of the Myb308 Phenotype

Overexpression of the Antirrhinum *AmMYB308* and *AmMYB330* genes in tobacco could affect transcriptional control in several ways. The introduction of transcription factors from another species might interfere with the functioning of the endogenous regulators, creating dominant inhibitors if, for example, the Antirrhinum proteins could bind to the same target motifs as the homologous tobacco proteins but be unable to transactivate target gene transcription normally. We constructed a truncated version of the *AmMYB308* gene by removing sequences encoding part of a conserved region within the potential activation domain of the AmMYB308 protein to test this possibility (Figures 1A and 1D). The stability of this protein product was confirmed by expression in *Escherichia coli.* None of the 50 transformants carrying this construct showed the Myb308 phenotype, indicating that AmMYB308 was not acting by inhibiting the functions of a tobacco homolog.

Alternatively, AmMYB308 and AmMYB330 could exert their effects in tobacco by binding nonspecifically to another tobacco transcription factor, which would then be inhibited in its action (Ptashne, 1988; Foos et al., 1992). We reasoned that this was not likely because overexpression of the other four Antirrhinum *MYB* genes (Jackson et al., 1991) in tobacco did not give rise to the Myb308/Myb330 phenotype (F.A. Culianez-Macia, S. Mackay, and C. Martin, unpublished results). To confirm this interpretation, we made a chimeric construct incorporating the DNA sequence encoding the third MYB repeat (R3) of AmMYB305 (Jackson et al., 1991) instead of R3 of AmMYB308 (pJAM939; Figure 2E). The pJAM939 construct was designed to test the effect of modifying the AmMYB308 binding domain without significantly affecting its overall structure. Twenty-seven transgenic plants were regenerated carrying this construct. None of these plants had the Myb308 phenotype, showing that the Myb308 phenotype was dependent on a functional and a specific DNA binding domain.

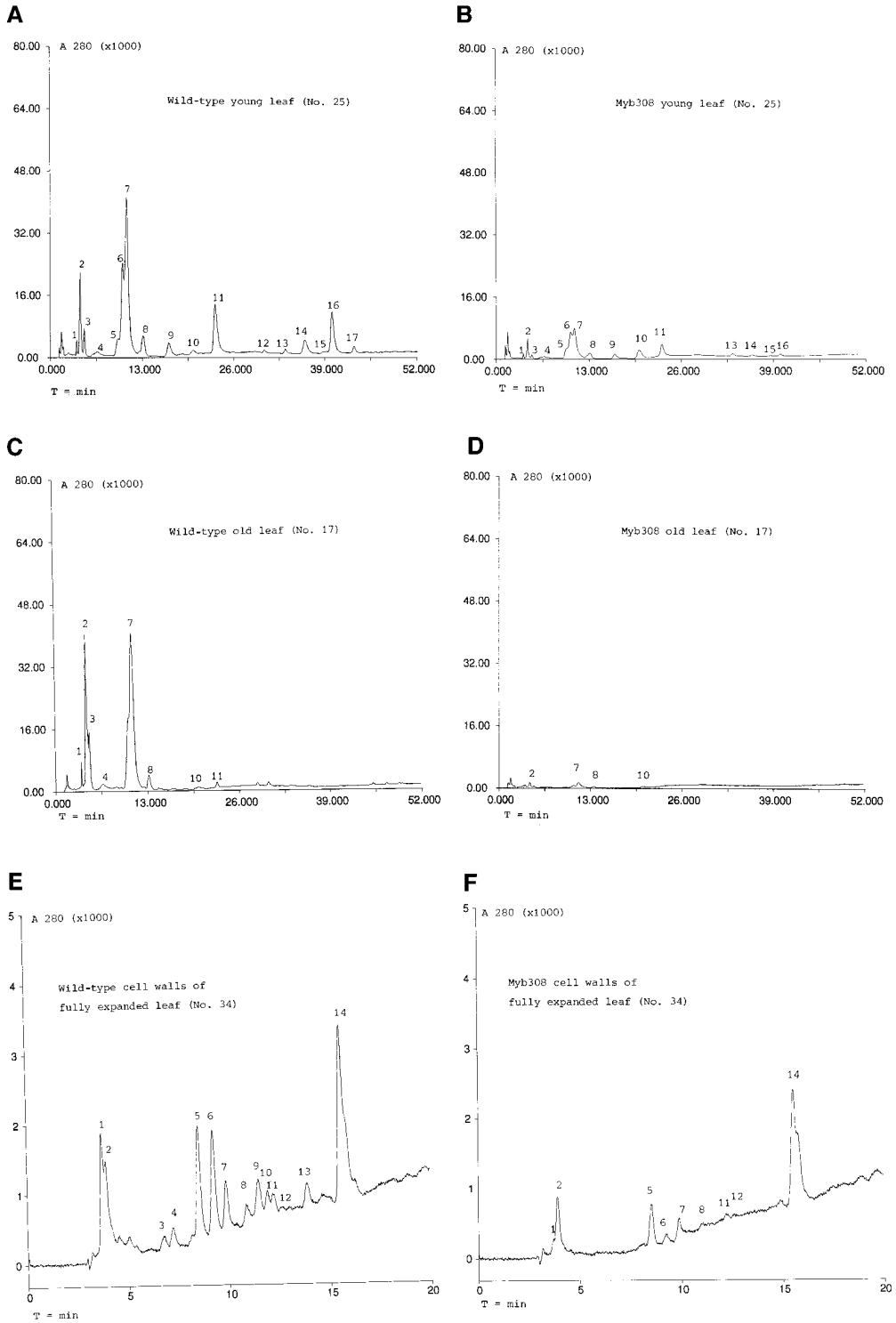

Figure 7. HPLC Analysis of Methanolic Extracts from Control and Myb308 Leaves of Equivalent Ages.

(A) Untransformed control, young leaves.
(B) Myb308, young leaves.
(C) Untransformed control, old leaves.

The C1 protein of maize (which is also a MYB domain transcription factor) interacts with a basic helix-loop-helix (bHLH) protein (R) to control expression of anthocyanin biosynthetic genes (Goff et al., 1992). To test whether AmMYB308 was titrating out a bHLH protein to bring about its phenotypic effects, we crossed Myb308 plants to tobacco plants ectopically expressing *DELILA* under the control of the CaMV 35S promoter (Mooney et al., 1995). The *DELILA* gene encodes a bHLH protein in Antirrhinum that controls anthocyanin biosynthesis (Martin et al., 1991; Goodrich et al., 1992). Plants expressing both *AmMYB308* and *DELILA* retained the Myb308 phenotype (as well as their Delila phenotype), suggesting that it did not result from titration of a bHLH protein.

Taken together, these results imply that the Myb308/Myb330 phenotypes arise from high levels of *AmMYB308*/*AmMYB330* expression, with their transcription factor products recognizing their normal target genes and repressing the expression of these genes as a consequence.

Table 1. Chlorogenic Acid Content of Control, Myb308, and Myb330 Plants[a]

Sample	Chlorogenic Acid (μg per g FWT)
Control (YL)	220.86
Myb308 (YL)	49.68
Control (ML)	215.66
Myb308 (ML)	8.27
Myb330 plant 1 (ML)	19.57
Myb330 plant 2 (ML)	19.54
AmMYB330 transformant plant 3 (ML)	273.91
AmMYB330 transformant plant 4 (ML)	268.92

[a] Myb330 plants 1 and 2 both showed strong phenotypic alterations and had high levels of *AmMYB330* gene expression. Plants 3 and 4 from the *AmMYB330* transformation showed no phenotypic alterations and did not express *AmMYB330*. All plants analyzed from the *AmMYB330* transformation were primary transformants. FWT, fresh weight of tissue; ML, mature leaf; YL, young leaf.

Transcriptional Control of the *4CL* Promoter by the AmMYB308 Protein in Yeast

To test whether the interaction between the AmMYB308 protein and the structural genes of phenolic metabolism was a direct one, the influence of AmMYB308 on transcription from potential target sequences present in the *4CL* promoter was tested in yeast. A 235-bp fragment upstream of the TATA box from the Antirrhinum *4CL* promoter was fused to a minimal *CYC1* promoter driving the β-galactosidase gene in the integrative plasmid pMH1. This fragment contains several putative MYB binding sites as well as the domain suggested to be responsible for repression of *4CL* expression in phloem tissue (Hauffe et al., 1993). The effector plasmid was obtained by introducing the *AmMYB308* gene under the control of the *GAL1* promoter in the pYES2 plasmid. Four independent yeast clones containing both the effector and reporter constructs were used for the β-galactosidase assays (Miller, 1972). Control values were obtained from yeast containing the reporter construct but without the *AmMYB308* gene in the effector plasmid. Data were calculated from the average of five independent assays. Values were 0.7 ± 0.05 units for AmMYB308 compared with 0.38 ± 0.03 units for the controls. This very low but reproducible activation from the *4CL* promoter suggested that AmMYB308 interacts directly with the structural genes involved in phenolic metabolism but that it is only a very weak activator of their transcription similar to the AmMYB305 protein of Antirrhinum (Moyano et al., 1996).

DISCUSSION

Overexpression of the AmMYB308 and AmMYB330 transcription factors in tobacco resulted in a complex phenotype in

Figure 7. (continued).

(D) Myb308, old leaves.

(E) Cell walls of untransformed control leaves (mature leaf).

(F) Cell walls of Myb308 leaves (mature leaf).

In **(A)** to **(D)**, peak 1 was not identified; peak 2 is an isomer of chlorogenic acid; peak 3 is an isomer of chlorogenic acid; peak 4 was not identified; peak 5 is an isomer of chlorogenic acid; peak 6 is an isomer of chlorogenic acid; peak 7 is chlorogenic acid; peak 8 is coumaroyl quinate; peak 9 was not identified; peak 10 is a tryptophan derivative; peak 11 is caffeoyl putrescine; peak 12 is a dihydroxycinnamic acid or a hydroxymethoxycinnamic acid ester; peak 13 is rutin; peak 14 was not identified; peak 15 is a flavonoid; peak 16 is a dihydroxycinnamic acid or a hydroxymethoxycinnamic acid amide; and peak 17 is a dihydroxycinnamic acid or a hydroxymethoxycinnamic acid ester.

In **(E)** and **(F)**, peak 1 was not identified; peak 2 was not identified; peak 3 was not identified; peak 4 was not identified; peak 5 is 4-hydroxybenzaldehyde; peak 6 was not identified; peak 7 is vanillin; peak 8 was not identified; peak 9 was not identified; peak 10 is 4-coumaric acid; peak 11 was not identified; peak 12 is ferulic acid; peak 13 was not identified; and peak 14 was not identified.

Leaves sampled were counted from the bottom of the plant. Leaf 17 was an older leaf than leaf 25 on the same plant. Samples for cell wall analysis were taken from older plants when leaf 34 was fully expanded. Typically, plants had between 33 and 38 leaves before flowering.

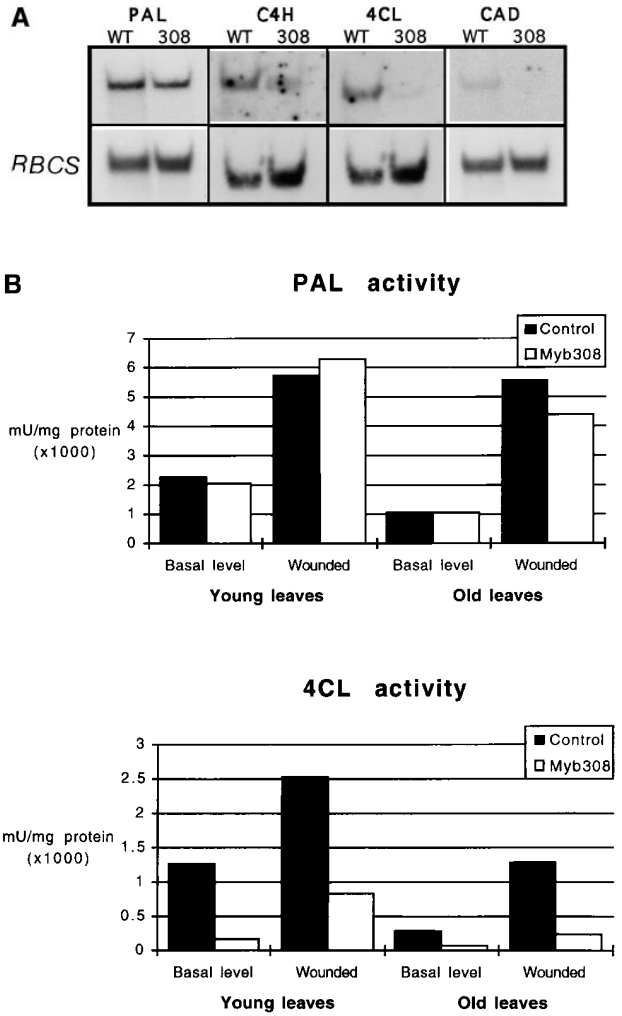

Figure 8. RNA Gel Blot Analysis of Expression of Phenolic and Lignin Biosynthetic Genes.

(A) No difference was observed in the expression of the gene encoding PAL between wild-type (WT) and Myb308 (308) tobacco in 15 μg of poly(A)$^+$ RNA prepared from leaves. Transcripts of *4CL* and *CAD* were almost undetectable in samples compared with samples from control leaves (WT). The *C4H* transcript was still present in Myb308 leaves but at a lower level than in wild-type leaves. Equal amounts of RNA were compared, as shown by the internal control *RBCS.* The results shown are representative of assays repeated three times.

(B) PAL and 4CL activity assays in control and Myb308 plants. The basal level of 4CL activity was clearly affected by the expression of *AmMYB308.* However, wounding still induced the activity of this enzyme. The activity of PAL (either basal or induced by wounding) was not affected in the samples from Myb308 leaves when compared with controls. mU, microunits.

the regenerated plants and in their progeny. The most striking effects were seen in leaves, but the overall growth rate of the plants was also significantly reduced. Although the production of lignin was dramatically reduced in Myb308 plants, the morphology of the vascular tissue appeared relatively normal. These phenotypic changes were observed in a significant number of primary transformants and were inherited as a dominant trait that cosegregated with the *AmMYB308* transgene (or the *AmMYB330* transgene) and the neomycin phosphotransferase II marker conferring kanamycin resistance. Moreover, comparison of the effects of overexpression of *AmMYB308* with the effects of modified versions of the *AmMYB308* gene showed that the phenotypic changes required the specific DNA binding domain of AmMYB308 as well as its C-terminal domain. AmMYB330, which has an almost identical DNA binding domain to AmMYB308, gave rise to an almost identical phenotype in transgenic plants, indicating that the two genes have basically the same set of target genes and very similar functions.

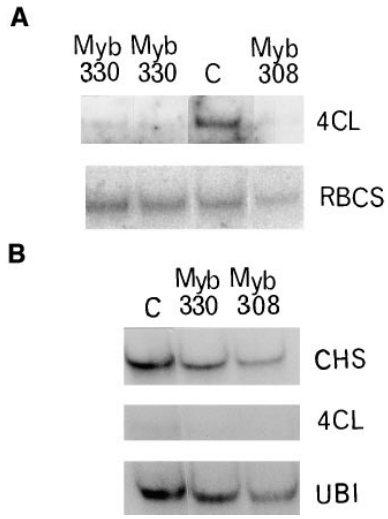

Figure 9. Expression of the Gene Encoding 4CL in Wild-Type and Myb308 and Myb330 Transgenic Lines.

(A) Poly(A)$^+$ RNA (5 μg) prepared from tobacco leaves was separated and probed with the tobacco *4CL* cDNA. Two independent Myb330 plants showing a modified phenotype showed a similar reduction in *4CL* expression to that observed in Myb308 plants. C indicates RNA from untransformed control plants. Loading of RNA in each track is indicated by hybridization with RBCS as an internal control.

(B) Effect of *AmMYB308* and *AmMYB330* on flavonoid gene expression. Total RNA prepared from tobacco flowers was probed with the tobacco *CHS* cDNA. A slight reduction in *CHS* transcript level (50% as estimated by using a PhosphorImager [model Fujix Bas 1000; Fuji, Tokyo, Japan]) was observed in Myb308 flowers, relative to untransformed control flowers (C) or Myb330 flowers, when hybridization was compared with the ubiquitin standard (UBI). The expression of the gene encoding 4CL was also reduced in Myb308 and Myb330 flowers, as previously observed in leaves.

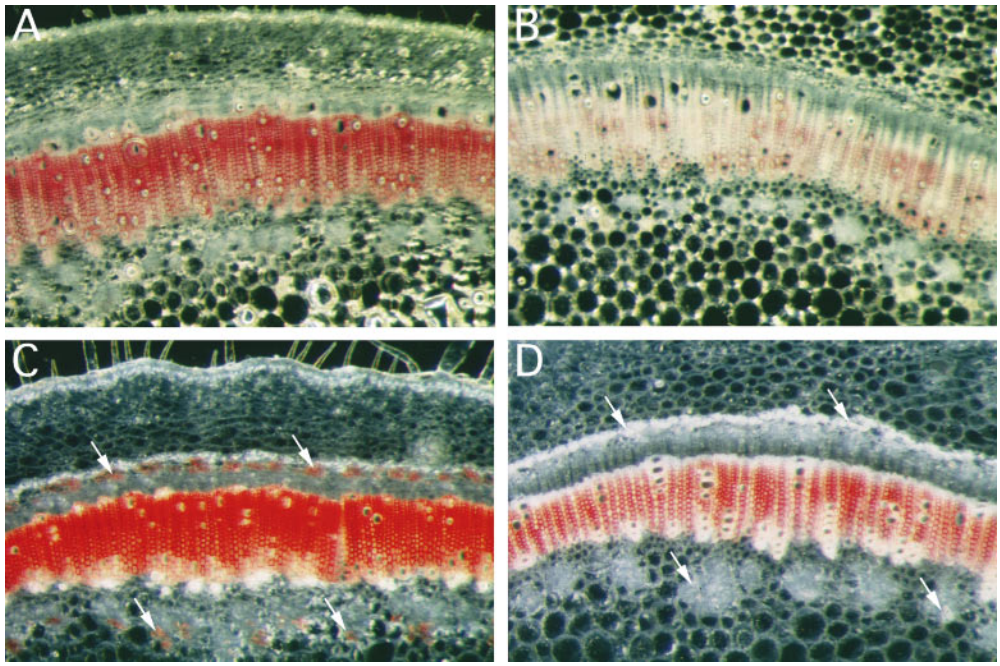

Figure 10. Histochemical Staining of Lignin.

(A) Control stem stained with phloroglucinol.
(B) Myb308 stem stained with phloroglucinol. Note the faint and irregularly distributed staining. The architecture of the wood does not seem to be affected by *AmMYB308* overexpression; however, the parenchyma cells between the epidermis and the cambium appeared enlarged in all sections.
(C) Control stem stained with the Mäule reagent. Note the positive reaction on the inner and outer rings of the phloem fibers (arrows).
(D) Myb308 stem stained with the Mäule reagent. Note the complete absence of staining on the inner and outer rings of phloem fibers (arrows). Note also the bigger size of the parenchyma cells.

The severity of the Myb308 phenotype correlated well with the quantity of the *AmMYB308* transcript, suggesting that the actual cellular concentration of the AmMYB308 protein is important for the phenotypic changes. Similar correlations between the severity of the phenotype and transgene expression were observed for *AmMYB330.* Extensive biochemical analyses revealed obvious qualitative differences only in the phenolic make-up of the Myb308 and Myb330 plants when compared with control plants. No obvious qualitative differences were found in total protein extracts from leaves (either soluble or from cell walls), in total protein profiles derived from in vitro translation of mRNA from leaves, or in cell wall carbohydrates in leaves, although quantitative differences were found in the dry weight of cell wall material between wild-type and Myb308 plants, which probably resulted from the direct effects on phenolic metabolism.

We believe that inhibition of production of phenolic compounds is the primary effect of overexpression of both *AmMYB308* and *AmMYB330.* This is supported by several additional lines of evidence. First, the inhibition of phenolic production occurs in seedlings and other very young tissue (as visualized under UV light and by phenolic extraction and HPLC analysis) before many of the cellular and physiological aspects of the phenotype are visible. Second, the Myb308 (and Myb330) phenotypic changes are very similar to the effects reported for inhibition of PAL activity by cosuppression in transgenic tobacco (Elkind et al., 1990). Inhibition of the activity of PAL would also limit phenolic production and should give rise to similar phenotypic changes in leaves of tobacco. Third, the involvement of *AmMYB308* and *AmMYB330* in phenolic production correlates well with the function of other *MYB* genes, which control the activity of various branches of phenylpropanoid biosynthesis (Paz-Ares et al., 1987; Avila et al., 1993; Grotewold et al., 1994; Sablowski et al., 1994; Solano et al., 1995; Moyano et al., 1996). We believe that both *AmMYB308* and *AmMYB330* might actually encode weak activators/repressors that compete with other stronger MYB-related activators to control target gene

Table 2. Composition of G and S Units in Lignin from Stems of Myb308 and Control Plants

	Control		Myb308	
Characteristics[a]	(G) Vanillin	(S) Syringaldehyde	(G) Vanillin	(S) Syringaldehyde
Top of stem (μg per g FWT)	2.51	2.74	2.48	1.82
% G/S		48:52		58:42
Bottom of stem (μg per g FWT)	37.05	66.92	13.14	34.66
% G/S		36:64		27:73

[a] Stems were harvested from the top and bottom of three tobacco plants that were ~1 m high and grown in a greenhouse. The composition of G and S units in lignin from stem samples of Myb308 and control plants after nitrobenzene oxidation is shown. FWT, fresh weight of tissue.

expression, as has been suggested for B-*MYB* from chicken (Foos et al., 1992) and perhaps B-*MYB* in mouse (Watson et al., 1993).

Differential Effects on Phenolic and Monolignol Metabolism

RNA gel blot analysis showed that two genes encoding enzymes of general phenylpropanoid metabolism (4CL and C4H) and one in the lignin branch (CAD) were affected by *AmMYB308* at the transcript level (Figure 8). Therefore, this transcription factor is able to inhibit multiple steps in the pathway for phenolic acid/monolignol biosynthesis, and it seems possible that *AmMYB308* and *AmMYB330* repress the expression of genes encoding all of the enzymes that catalyze the steps between C4H and CAD. Many of these genes contain H-box– or P-box–like motifs in their promoter regions, which commonly are recognition sites for MYB protein binding (Grotewold et al., 1994; Sablowski et al., 1994; Solano et al., 1995; Douglas, 1996; Martin and Paz-Ares, 1997). Interestingly, functional negative regulatory regions have been described for the promoter of a *4CL* gene from parsley (Hauffe et al., 1993). This gene carries motifs similar to the sequences of other plant MYB binding sites (Grotewold et al., 1994; Sablowski et al., 1994). These could be the target site for AmMYB308/AmMYB330 binding.

Our data support the idea of coordinate regulation of genes involved in phenylpropanoid metabolism by common regulatory proteins, as originally suggested by Hahlbrock et al. (1976). However, *AmMYB308* does not affect the steady state levels of *PAL* transcripts, suggesting that the *PAL* genes are not included as targets for joint regulation along with the other steps in the pathway by AmMYB308 and AmMYB330. *PAL* genes are responsive to other MYB domain transcription factors (Sablowski et al., 1994, 1995), and the coordination of expression of the *PAL* gene with the other biosynthetic genes could result from coordinate regulation of the different MYB factors in response to common signals.

Activity of C4H, 4CL, and CAD is essential for the production of the lignin precursors 4-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. Our evidence suggests that the silencing action of the AmMYB308 and AmMYB330 proteins on the corresponding target genes is the cause of the severe reduction in lignin that was observed in these transgenic plants. It is noteworthy that lignin levels were reduced by the action of transcription factors rather than by modifying the expression of structural genes. The results of phloroglucinol staining of lignin showed a large difference in the intensity of reaction of control and Myb308 xylem tissue, suggesting a conspicuous reduction in the amount of lignin in the transgenic plants. The production of coniferyl aldehyde units is strongly affected by *AmMYB308* overexpression. Staining of the xylem in sections from Myb308 stems with the Mäule reagent in ten lines examined showed that the coloration never reached the same intensity as wild-type samples, indicating that the production of S units (Nakamo and Meshitsuka, 1992) is also affected by AmMYB308 overexpression. The internal and external phloem fibers in wild-type stems seem to contain predominantly S-type lignin because they stain with the Mäule reagent but not with the phloroglucinol–HCl reaction. The phloem fibers of Myb308 stems seem to be devoid of lignin. They do not stain with either phloroglucinol or the Mäule reagent.

The data obtained from the alkaline nitrobenzene oxidation of lignin support the results of the histochemical staining. However, nitrobenzene degrades only those moieties within the polymer that are not involved in interunit aromatic carbon–carbon linkages (Lewis and Yamamoto, 1990; Chen, 1992). Therefore, the results cannot be considered as fully quantifying the effects of *AmMYB308* on lignin production. The change in ratio between G and S residues in lignin between control and Myb308 plants could have arisen from several causes. It is possible that AmMYB308 selectively inhibits expression of the different genes involved in monolignol production (compare with the results of Atanassova et al. [1995], for example). Alternatively, AmMYB308 might cause equal inhibition of the individual steps, but under such circumstances, the branch leading to coniferyl alcohol might be more efficient at channeling limited precursors than the branch leading to sinapyl alcohol. Alternatively, AmMYB308 may repress lignin biosythesis more effectively in some tissues than in others. The change in G/S composition might then reflect the contribution of such different cell types to total lignin composition. These possibilities will be tested in a more detailed analysis of the effects of AmMYB308 on monolignol biosynthesis.

In the industrialized nations, a major fraction of harvested wood is processed for pulp and papermaking. This process requires the removal of most of the lignin by using harsh chemical treatments that are expensive and environmentally unfriendly (Dixon et al., 1994; Sederoff et al., 1994). There is

also interest in lowering the content of lignin in forage crops to improve digestibility (Iiyama et al., 1993). In recent years, there have been several attempts to reduce lignin quantity by reducing the activity of structural genes along the pathway. This approach has produced striking changes in lignin quality, but the reduction in overall lignin content has not been conspicuous (see, e.g., Dwivedi et al., 1994; Halpin et al., 1994; Ni et al., 1994; Atanassova et al., 1995; Hibino et al., 1995; Van Doorsselaere et al., 1995). The use of transcription factors, such as AmMYB308 or AmMYB330, for this purpose could potentially reduce lignin production, providing that a suitable promoter can be found to drive gene expression. Ideally, such a promoter should be xylem specific and should be activated only during lignin formation, thereby avoiding problems affecting plant growth and development that arise as a consequence of a generalized reduction in phenolic compounds, as observed in the premature cell death in Myb308 and Myb330 plants. One possibility would be to use the *gPAL2* promoter from bean. Parts of this promoter could be used to drive expression specifically in xylem (Sablowski et al., 1994; Hatton et al., 1995).

In summary, the overexpression of *AmMYB308* and *AmMYB330* inhibited production of the end products of phenylpropanoid metabolism and its side branches. The production of lignin and soluble phenolic acid esters was affected the most. Flavonoid production, however, was considerably less affected. This establishes another branch of phenylpropanoid metabolism as being under the control of a distinct subclass of MYB proteins (cf. Grotewold et al., 1994; Sablowski et al., 1994; Moyano et al., 1996). Our results indicate that a generalized deficiency in phenolic compounds leads to an array of physiological changes in transgenic plants. Among these is a reduction in the production of lignin. The other phenotypic changes in Myb308 and Myb330 plants indicate subsidiary functions of phenolic compounds in tobacco, concerned with cellular development and senescence. These additional physiological effects of modified phenolic metabolism will be described in a separate study (L. Tamagnone, A. Merida, N. Stacey, K. Plaskitt, D. Lynn, J.M. Dow, K. Roberts, and C. Martin, manuscript in preparation).

METHODS

Plant Material and Transformation

Tobacco (*Nicotiana tabacum* cv Samsun NN) plants were transformed with *Agrobacterium tumefaciens* LBA4404 (Ooms et al., 1982), following the method of Horsch et al. (1985). Once rooted on selective medium (100 μg mL^{-1} kanamycin), shoots that were $\sim$5 cm high were transferred to peat compost and grown to maturity in the greenhouse.

Plants were allowed to set self-fertilized seed. Seeds were surface sterilized in Domestos (Lever Bros., Kingston upon Thames, UK), washed in sterile distilled water, and sown on Murashige and Skoog agar (Murashige and Skoog, 1962) with kanamycin (100 μg mL^{-1}) selection. Selected seedlings were potted into peat and grown to maturity in the greenhouse. Leaves harvested for phenolic assays were divided into young and older leaves. Comparable leaves were taken from control and Myb308 plants by counting the number of leaves from the bottom of the tobacco plant. Tobacco (Samsun NN) expressing *DELILA* under the control of the cauliflower mosaic virus (CaMV) 35S promoter was a generous gift from M. Mooney (Mooney et al., 1995).

Overexpression Constructs of *AmMYB308* and *AmMYB330*

We used in vitro mutagenesis to introduce an NcoI site over the first ATG in the *AmMYB308* cDNA coding sequence (Jackson et al., 1991). The mutagenized cDNA clone was cut with NcoI and EcoRI (which cuts at the 3′ end of the *AmMYB308* cDNA), and the fragment was cloned into the plant expression cassette of pJIT166 (Guerineau and Mullineaux, 1993) cut with NcoI and EcoRI. This construct was fused to the *AmMYB308* cDNA sequence in frame with the first ATG after the transcriptional start of the double CaMV 35S promoter sequence. The untranslated leader sequence of the *AmMYB308* gene in this construct contains an eukaryotic ribosome binding site (Kozak, 1983). The CaMV terminator sequence (derived from pJIT166) was included at the 3′ end of the *AmMYB308* cDNA to ensure transcriptional termination. This construct was called pJAM495 (Figure 2B).

A similar construct was built for the expression of *AmMYB330.* The full-length cDNA was cloned into the EcoRI site of pJIT60 (Guerineau and Mullineaux, 1993) in the sense orientation. The leader sequence in the cDNA contains a sequence that conforms well to the consensus for translational initiation around the first ATG (Kozak, 1983). The CaMV terminator was used 3′ to the *AmMYB330* coding sequence. This plasmid was called pJAM355 (Figure 2F).

Antisense Expression Constructs of *AmMYB308* and *AmMYB330*

The *AmMYB308* cDNA was cut with SspI, which cuts just upstream of the first ATG of the *AmMYB308* coding sequence, and with XhoI, which cuts in the polylinker at the 3′ end of the *AmMYB308* cDNA insert in pBluescript SK+ (Stratagene, La Jolla, CA). This fragment was cloned between the SalI and SmaI sites of pJIT60 (Guerineau and Mullineaux, 1993), which lie in a polylinker between the double CaMV 35S promoter sequences and the CaMV terminator, to give antisense expression of *AmMYB308.* This construct was called pJAM497 (Figure 2C). The *AmMYB330* cDNA was cloned in the antisense orientation into the EcoRI site of the polylinker of pJIT60. This plasmid was called pJAM354 (Figure 2G).

Truncated *AmMYB308* Gene Construct

As a control for the effect of overexpression of *AmMYB308*, part of the sequence encoding the potential activator domain of the transcription factor was removed. The deduced amino acid sequence between amino acids 151 and 188 of the AmMYB308 protein is the region most likely to form an amphipathic α-helix, according to the Chou-Fasman algorithm (Chou and Fasman, 1978). A motif from the predicted amino acid sequence of AmMYB308 (amino acids 176 to 188), which lies within the region of amphipathic α-helix (amino acids 151 to 188), is highly conserved in a number of *MYB* genes from

different plants (Marocco et al., 1989; Jackson et al., 1991), supporting the view that this region is required for the function of the AmMYB308 protein.

The cDNA sequence of the region encoding this conserved motif (from base pairs at positions +528 to +564) contains a BsaBI site (+536). Construct pJAM495 was cut with BsaBI and EcoRI to remove the sequence encoding most of the conserved motif plus the remaining sequence encoding the AmMYB308 C terminus and the 3′ trailer sequence. The EcoRI site was filled in and religated to give a construct that would express a truncated AmMYB308 protein lacking amino acids 179 to 232. This construct was called pJAM933 (Figure 2D). The NcoI-EcoRI fragment of pJAM495 was cloned into pRSETB (Invitrogen Corp., San Diego, CA) and expressed in *Escherichia coli* K38, as described in Edwards et al. (1995). The plasmid was cut with BsaBI and EcoRI and religated, as was done in the construction of pJAM933. This truncated construct was also expressed in *E. coli* to confirm the stability of the truncated protein. Production of the protein was readily detected at similar or higher levels than was the full-length AmMYB308 protein expressed under identical conditions.

Modified AmMYB308 DNA Binding Domain

The *Antirrhinum majus* transcription factor AmMYB308 does not bind to the cognate c-MYB binding motif TAACGG (Biedenkapp et al., 1988) in vitro, whereas the Antirrhinum transcription factor AmMYB305 binds weakly to this sequence (Jackson et al., 1991; F.A. Culianez-Macia and C. Martin, unpublished results). This implies that these *MYB* gene products have different affinities for particular target motifs. To modify the DNA binding properties of the *AmMYB308* product, we exchanged part of the sequence encoding the DNA binding domain of AmMYB308 for the matching region of AmMYB305. Restriction enzyme sites were introduced by in vitro mutagenesis into the *AmMYB305* and *AmMYB308* cDNA sequences so that the sequences encoding amino acids 65 to 117 in *AmMYB305* and 64 to 116 in *AmMYB308* (structurally equivalent to the third repeat, R3, of the mammalian c-MYB DNA binding domain; Paz-Ares et al., 1987; Jackson et al., 1991) could be exchanged. The amino acid changes involved in the creation of these restriction sites were either conservative or outside of the region believed to be involved in DNA binding (in AmMYB305, amino acid 65 V→I and amino acid 117 M→V; in AmMYB308, amino acid 117L→N). The modified construct was cloned between the double CaMV 35S promoter and terminator of pJIT166 in the same way as in the construction of pJAM495; the resulting plasmid was called pJAM939 (Figure 2E).

The gene constructs in pJAM343, pJAM344, pJAM354, pJAM355, pJAM495, pJAM497, pJAM933, and pJAM939 were isolated from their plasmids by cutting with KpnI and XhoI, and each was cloned between the KpnI and SalI sites of pBIN19 (Bevan, 1984). These binary vectors were transferred to Agrobacterium by triparental mating, using the helper plasmid pRK2013. The structure of each construct in *E. coli* and Agrobacterium was confirmed by restriction mapping to ensure that rearrangement had not occurred.

Extraction of DNA and RNA

DNA was extracted from leaves of tobacco, according to Martin et al. (1985). DNA gel blot hybridizations were performed according to Southern (1975) and Wahl et al. (1979), as modified by Sommer et al. (1985). Total RNA was extracted from tobacco leaves, as described by Prescott and Martin (1987), analyzed by electrophoresis in formaldehyde gels, and transferred to nitrocellulose.

Observation of Leaves under UV Light

Young and mature leaves of wild-type and Myb308 plants were illuminated with a direct source of UV light (365 nm) coming from two transilluminators placed at an angle of 45° on each side of the leaves. Photographs were taken using a Wratten No. 8 filter (Kodak, Hemel Hempsted, UK) to remove the scattered UV light.

Analysis of Phenolics

Fresh leaf material was frozen in liquid N_2 and ground to a fine powder. Dry ice-cold methanol (AR, for analytical reagent grade) was added in the ratio of 2.7 mL per gram of leaf material. The powder was resuspended by vigorous shaking and extracted with additional mixing for 30 min on dry ice. The mixture was centrifuged at 50*g* for 5 min at 4°C, and the pellet, which was composed mainly of cell walls, was stored frozen and used for analysis of cell wall–bound phenolics (see below). The supernatants were clarified by centrifugation at 2000*g* for 30 min at 4°C and then analyzed by HPLC in a 60-min run, using a gradient of increasing methanol in conjunction with a Spherisorb ODS2 3-μm column (Fisons Chromatography, Loughborough, UK) or, alternatively, a C18 column (Waters μBondapak; Millipore Corp., Bedford, MA). The flow rate was 1 mL/min. Solvents were as follows: A, 10% MeOH, 90% (v/v) H_2O, 1 mM trifluoroacetic acid (TFA); B, 80% MeOH, 20% (v/v) H_2O, 1 mM TFA. The gradient was 90% solvent A and 10% solvent B at time 0, 50% solvent A and 50% solvent B at 40 min, and 100% solvent B at 60 min.

Cell wall pellets were washed three times with ice-cold 80% ethanol and once with deionized water by successive centrifugation and resuspension. Phenolics esterified to the cell walls were hydrolyzed with 1 M NaOH for 1 hr at room temperature. The mixture was brought to pH 3 by the addition of 1 M HCl, and the protonated phenolic acids were then extracted twice with ether. The ether was left to evaporate in a fume hood, and the phenolic residue was resuspended in 70% methanol (AR) in a ratio of 0.9 mL per gram fresh weight of the original sample. The methanolic solution was used directly for HPLC, with a more compressed linear gradient (25 min) used in conjunction with an Inertpak ODS2 Ik5 column (Capital HPLC, Broxburn, UK). The flow rate was 1 mL/min, and 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 Spectra Physics (San Jose, CA) HPLC apparatus was used for all analyses, and the column effluent was monitored using a Spectra FOCUS (Spectra Physics Ltd.) scanning detector to provide full spectral information on each peak as it was eluted.

Samples were kept cold throughout the procedure to avoid oxidation from possible residual peroxidase activity in the methanolic solution. Samples were also kept away from direct sunlight or strong light sources to avoid isomerization.

Some of the separated compounds (e.g., chlorogenic acid, caffeoyl putrescine, rutin, and vanillin) were identified on the basis of their having identical HPLC behavior and UV absorption spectra to known standards. Those compounds, defined only by class (e.g., fla-

vonoids, dihydroxycinnamic acid esters, and amides), were assigned on the basis of characteristic UV spectra.

In all, soluble phenolics were analyzed in eight different wild-type lines, including lines transformed with the binary vector pBin19 and untransformed Samsun lines. Soluble phenolics were analyzed in seven extractions from two independent Myb308 lines. Cell wall phenolics were analyzed in five separate extractions from wild-type plants (including plants transformed with pBin19 and untransformed Samsun) and in five separate extractions from two independent Myb308 lines. Essentially the same differences were observed between all Myb308 and all control extracts.

Histological Staining of Lignin

Cross-sections of stem of 10 Myb308 plants and 10 control plants were cut using a vibrotome set at a thickness of 7 μm. Sections were immediately placed on glass microscope slides and kept wet by adding distilled water. For phloroglucinol staining, after removing the water, we applied a few drops of phloroglucinol solution directly to the specimens, which were left to react for 3 min. After this time, a few more drops of the phloroglucinol solution were added and a coverslip was placed on the slides. Photographs were taken within 30 min using a Zeiss (Jena, Germany) dissecting microscope fitted with a camera. For staining with the Mäule reagent, the water was removed and sections were stained with Mäule reagent, according to Chapple et al. (1992). Because the intensity of both reactions is time dependent, control and Myb308 samples were observed together on the same slide for comparison.

Nitrobenzene Oxidation of Lignin

This method was devised following those of Galletti et al. (1989), Chapple et al. (1992), and Ni et al. (1994). Stems of three plants were divided into the top and bottom portions, to distinguish between newly formed lignin and more mature lignin. The samples were frozen in liquid N_2 and ground to a fine powder. One gram of the powder was washed three more times with methanol to remove residual soluble phenolics and then twice with deionized water at 60°C. Esterified phenolics were saponified with 1 M NaOH at 37°C for 1 hr, and then the residue was washed twice with deionized water. Each pellet was resuspended in 8 mL of 2 M NaOH and 200 μL of nitrobenzene, and the pyrex tubes were sealed and heated to 165°C for 2 hr. After cooling, the reaction products were acidified to pH 3 with HCl and partitioned into dichloromethane. The organic phase was collected and dried by heating at 50°C under a stream of nitrogen. The residue was resuspended in 0.5 mL of methanol (AR) and analyzed by HPLC, as was described for the cell wall–bound phenolics.

Phenylalanine Ammonia–Lyase and 4-Coumaroyl–CoA Ligase Assays

Phenylalanine ammonia–lyase (PAL) activity was assayed according to Rhodes and Wooltorton (1971), after the absorbance of cinnamic acid at 290 nm. 4-Coumaroyl–CoA ligase (4CL) activity was assayed according to Rhodes and Wooltorton (1973), after the synthesis of the product 4-coumaroyl–CoA at 333 nm.

Isolation of Tobacco 4CL and C4H cDNA Clones

A cDNA library in λgt10 made from mRNA from tobacco leaves was screened with a DNA fragment encoding the potato 4CL enzyme (Fritzemeier et al., 1987). From 12 positive clones, the one with the largest insert (1.7 kb) was selected, and its identity was confirmed by sequencing. The library was also screened with the cDNA clone encoding cinnamate 4-hydroxylase (C4H) from artichoke (Teutsch et al., 1993). From the positive clones, the one with the longest insert (2 kb) was selected, and its identity was confirmed by sequencing. A probe for the gene encoding cinnamyl alcohol dehydrogenase (*CAD*) from tobacco was obtained from a plasmid generously provided by Wolfgang Schuch (Zeneca Seeds, Bracknell, UK).

Cloning of Region Upstream of the *4CL* Gene from Antirrhinum

An EMBL4 genomic library from Antirrhinum was screened using the tobacco *4CL* gene as a probe. Six positive clones were isolated and showed overlapping fragments by restriction analysis. One of those cloned was used to isolate a 1.3-kb EcoRI fragment containing the 5′ coding sequence and the first 500 bp of the region upstream of the *4CL* gene. The sequence of this fragment was determined and has EMBL accession number Y15607.

Transcriptional Activation Assays in Yeast

A reporter plasmid was obtained by modification of pMH1 (Sablowski et al., 1994), which contains a truncated *CYC1* promoter fused to the *lacZ* reporter gene. A fragment of the region upstream of the gene *4CL* (extending from the first nucleotide 5′ to the TATA box upstream for 235 bp) was generated by polymerase chain reaction amplification and inserted by using engineered BamHI and XhoI restriction enzyme sites into the XhoI and BglII sites lying upstream of the truncated *CYC1* promoter. The segment generated by polymerase chain reaction was checked by sequencing. An effector plasmid was derived from pYES2 (Invitrogen Corp.) containing the *GAL1*-inducible promoter for expression of inserted genes. The effector plasmid contained the complete coding sequence of *AmMYB308.* Yeast cells (*Saccharomyces cerevisiae* JB811; *ura3-52 leu2 trp1*) were transformed with linearized reporter plasmid, according to Gietz et al. (1992). Plasmid integration was confirmed by DNA gel blot analysis. Four transformants for the reporter plasmid were used for a second transformation with the effector plasmid. Transformants were grown in liquid medium to an A-600 nm of 1.2 to 1.4, and GAL1 activity was assayed and enzyme units calculated according to Miller (1972).

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