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Arabidopsis MYB26/MALE STERILE35 Regulates Secondary Thickening in the Endothecium and Is Essential for Anther Dehiscence [W] [OA]

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The *Arabidopsis thaliana MYB26/MALE STERILE35* (*MS35*) gene is critical for the development of secondary thickening in the anther endothecium and subsequent dehiscence. MYB26 is localized to the nucleus and regulates endothecial development and secondary thickening in a cell-specific manner in the anther. *MYB26* expression is seen in anthers and also in the style and nectaries, although there is no effect on female fertility in the *ms35* mutant. *MYB26* expression in anthers occurs early during endothecial development, with maximal expression during pollen mitosis I and bicellular stages, indicating a regulatory role in specifying early endothecial cell development. Overexpression of *MYB26* results in ectopic secondary thickening in both *Arabidopsis* and tobacco (*Nicotiana tabacum*) plants, predominantly within the epidermal tissues. MYB26 regulates a number of genes linked to secondary thickening, including *IRREGULAR XYLEM1* (*IRX1*), *IRX3*, *IRX8*, and *IRX12*. Changes in expression were also detected in two NAC domain genes, *NAC SECONDARY WALL–PROMOTING FACTOR1* (*NST1*) and *NST2*, which have been linked to secondary thickening in the anther endothecium. These data indicate that MYB26 regulates *NST1* and *NST2* expression and in turn controls the process of secondary thickening. Therefore, MYB26 appears to function in a regulatory role involved in determining endothecial cell development within the anther and acts upstream of the lignin biosynthesis pathway.

INTRODUCTION

In *Arabidopsis thaliana*, as in most flowering plant species, the anther wall comprises four cell layers: the epidermis, endothecium, middle layer, and tapetum (Dawson et al., 1993). Anther dehiscence is a multiple-step process that includes the lytic opening of a longitudinal line of weakness in the epidermis, known as the stomium, and retraction of the anther wall to widen the stomium and permit pollen release. During microspore maturation, cellulose and lignified thickenings are deposited in the endothecium (Dawson et al., 1999). The cells of the stomium and circular cell cluster (present in tobacco [*Nicotiana tabacum*]) are then enzymatically lysed (Goldberg et al., 1993). The endothecium is thought to be critical in generating the forces required for dehiscence. First, swelling of the endothecium cells provides an inwardly directed force, causing the weakened stomium to rupture. Then, desiccation of the endothecium causes differential shrinkage of thickened and unthickened parts of the cell wall, resulting in an outwardly bending force, leading to retraction of the anther wall and full opening of the stomium (Keijzer, 1987; Bonner and Dickinson, 1989).

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A number of *Arabidopsis* dehiscence mutants have been characterized, such as *dde1/opr3* (Sanders et al., 2000; Stintzi and Browse, 2000), *coi1* (Feys et al., 1994; Xie et al., 1998), *dad1* (Ishiguro et al., 2001), and *aos/dde2-2* (Park et al., 2002; von Malek et al., 2002), which disrupt the jasmonic acid (JA) biosynthesis/signaling pathway. Analysis of these mutants suggests that water transport into the anthers is regulated by JA/ethylene (Ishiguro et al., 2001). The SUC1 protein, a plasma membrane H^+-sucrose symporter, accumulates in the connective cells surrounding the vascular tissue during the final stages of anther development, and it has been suggested that this causes an accumulation of sucrose in these tissues that results in increased water uptake (Stadler et al., 1999). It is speculated that JA may be needed for the expression of *SUC1* and other genes associated with water transport in anthers (Ishiguro et al., 2001).

The *ms35/myb26* mutation appears to disrupt a pathway distinct from that defined by the JA dehiscence mutants. Anther and pollen development follows the same pattern as in wild-type plants, with lysis of the cells forming the stomium, leaving no bridge between the epidermal/endothecial cells on either side of the stomium. However, wall thickenings are not formed in the *ms35* endothecium, resulting in the endothecial cells becoming flattened and distorted (Dawson et al., 1999). The lack of thickening means that the outward bending of the anther wall fails to occur, the endothecial cells collapse, and the anther fails to open. However, the pattern of lignification in the vascular tissue of the anthers, stems, and leaves is the same in wild-type and *ms35/myb26* plants. Therefore, the *ms35* mutation appears to specifically affect secondary wall deposition in the endothecium.

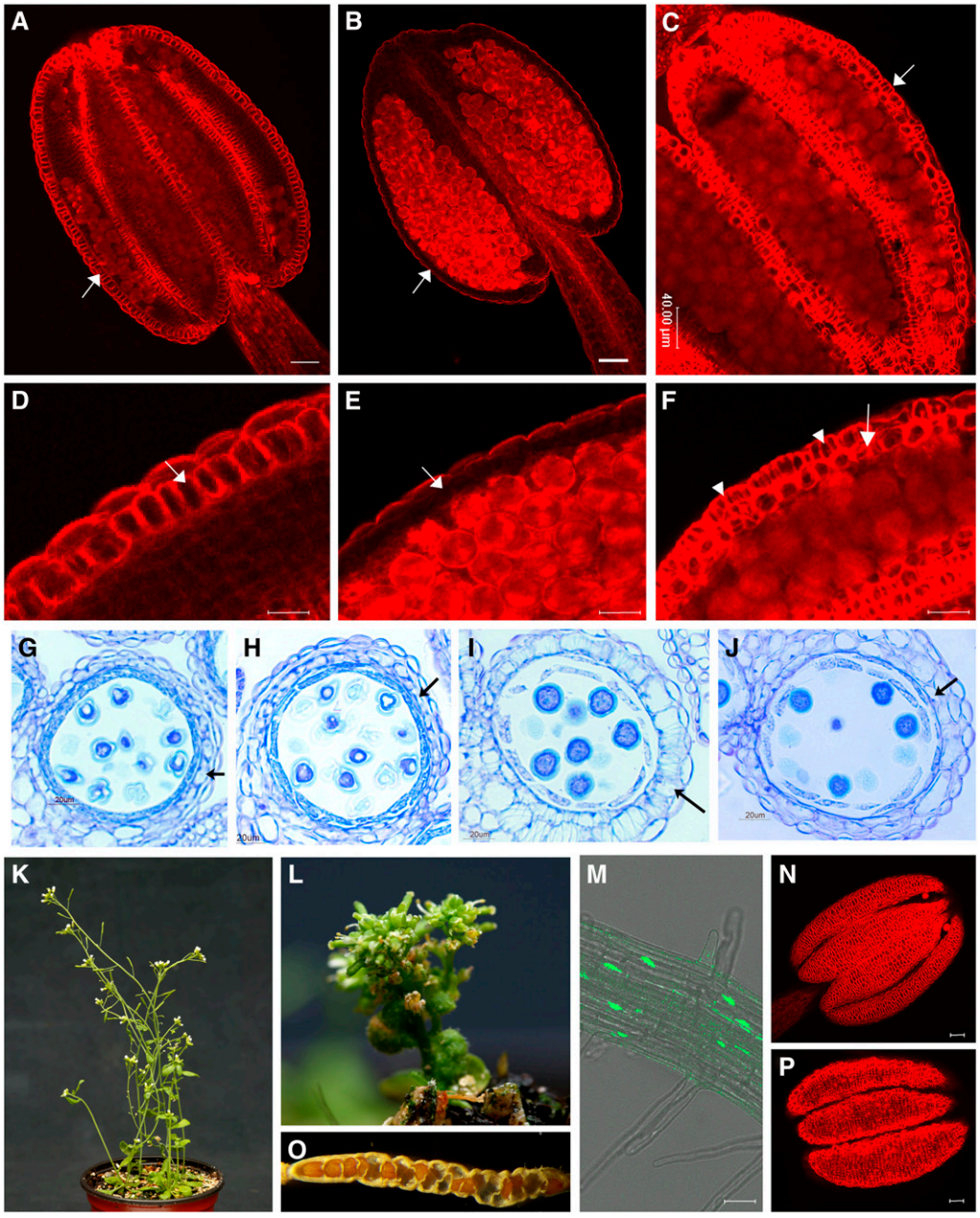

Figure 1. Phenotypic Characterization of *Arabidopsis ms35* Mutant and *Arabidopsis Pro35S:MYB26* Overexpression Lines.

(A) to **(F)** Anthers were stained with acridine orange/ethidium bromide and visualized by confocal microscopy (excitation, 590 nm).
(A) L*er* wild-type partially dehisced anther from open flowers. Secondary thickening is visible in the endothecium (arrow).
(B) *ms35* mutant nondehiscent mature anther from open flowers. Secondary thickening is absent in the endothecium (arrow).
(C) Mature anther from open flowers of a *MYB26* overexpression line.
(D) Close-up of **(A)**.
(E) Close-up of **(B)**.
(F) Close-up of **(C)**. Secondary thickening is visible in the endothecium (arrow) and as ectopic islands in the epidermal tissues (arrowheads).
(G) to **(J)** Sections through wild-type anthers (**[G]** and **[I]**) and *ms35* mutant anthers (**[H]** and **[J]**).
(G) During pollen mitosis I, endothecial cells begin to expand (arrow) in the wild type.
(H) At the corresponding stage in the *ms35* mutant, minimal endothecium expansion is seen (arrow).
(I) During pollen mitosis II, endothecium expansion continues in the wild type and secondary thickening is clearly evident (arrow).
(J) At the corresponding stage in the mutant, the endothecium cells fail to fully expand and no secondary thickening occurs (arrow).
(K) to **(M)** and **(O)** *MYB26* overexpression lines.

We have shown that the failure of dehiscence in the *ms35* mutant is attributable to a defect in the *MYB26* gene that is homologous with the R2R3-type MYB transcription factors (Steiner-Lange et al., 2003). R2R3-type MYB transcription factors are thought to act either as transcriptional activators or repressors, controlling development and the determination of cell fate as well as the regulation of biosynthetic pathways (Stracke et al., 2001). They have also been specifically linked to a variety of metabolic pathways associated with the regulation of phenylpropanoid metabolism (Borevitz et al., 2000; Yang et al., 2001; Patzlaff et al., 2003; Newman et al., 2004; Rogers and Campbell, 2004; Goicoechea et al., 2005; Rogers et al., 2005; Deluc et al., 2006). Overexpression of many of these MYB factors results in the induction of genes associated with the phenylpropanoid biosynthesis pathway; for example, in *Antirrhinum majus*, Am *MYB305* and Am *MYB340* regulate flavanol biosynthesis (Moyano et al., 1996), whereas Am *MYB308* (Tamagnone et al., 1998) and the *Arabidopsis* homolog At *MYB4* (Jin et al., 2000) downregulate hydrocinnamic acid and monolignol biosynthesis.

The deposition of lignified secondary thickening is usually only seen once cell growth has stopped. Secondary cell walls are composed predominantly of cellulose, lignin, and xylan, and the biosynthesis and deposition of wall material involves the coordinated regulation of a number of complex metabolic pathways. Defects associated with the failure of secondary thickening exhibit a diversity of phenotypes, ranging from collapse of the xylem vessels (*irregular xylem* [*irx*]) (Turner and Somerville, 1997) to loss of dehiscence (Mitsuda et al., 2005). Two NAC domain transcription factors, *NAC SECONDARY WALL–PROMOTING FACTOR1* (*NST1*) and *NST2*, have been proposed as regulators of secondary wall thickening and specifically endothecium thickening (Mitsuda et al., 2005). Mitsuda et al. (2005) proposed that NST1 and NST2 act redundantly to regulate secondary cell thickenings in anther walls and are required for anther dehiscence; they also implied that NST1 and NST2 may act by regulating *MYB26* expression.

We have performed a functional analysis of *MYB26* and demonstrated that MYB26 is localized to the nucleus and plays a critical role in the regulation of endothecial maturation and secondary thickening in a cell-specific manner in the anther. Overexpression of *MYB26* results in the development of ectopic secondary thickening in both *Arabidopsis* and tobacco plants. MYB26 appears to regulate a number of genes linked to secondary thickening. Changes in expression were detected in two NAC domain genes, *NST1* and *NST2*, and in *IRX1*, *IRX3*, *IRX8*, and *IRX12*, indicating that *MYB26* may be involved in their regulation. These data suggest that MYB26 plays a regulatory role in determining cell development, or competence for secondary thickening, within the anther and acts upstream of the lignin biosynthesis pathway, possibly via *NST1* and *NST2*.

RESULTS

Alterations in Secondary Thickening in the *ms35* Mutant

The *ms35* mutant is male-sterile as a result of a failure of anther dehiscence. In the wild type, secondary thickening occurs in the endothecium as bands of striated spring-like thickening that is composed of cellulose and lignin (Figures 1A, 1D, and 1N). Deposition commences during pollen mitosis I and is maximal by the end of pollen mitosis II (Figures 1G and 1I). Secondary thickening is absent from the surrounding cell layers of the anther and is necessary to create the shearing force required for anther dehiscence. In the *ms35* mutant, no secondary thickening or lignification is seen in the anther endothecium (Figures 1B and 1E), although other tissues in the mutant plant undergo normal secondary thickening. This lack of strengthening of the endothecium means that as the anther dehydrates the endothecium layer distorts, shearing forces do not develop, and the anther fails to open. The appearance of the endothecium changes before the onset of secondary thickening (Figure 1G). In the wild type, as the endothecial cells develop they expand, particularly toward the center of the locule. However, in the *ms35* mutant, the endothecial cell number does not appear changed, and these cells do not fully expand and do not have any secondary thickening (Figures 1H and 1J). Cell expansion is an essential prerequisite before laying down secondary thickening, because the rigidity of the secondary wall prevents subsequent expansion. This suggests that MYB26 acts at an early stage of endothecium development, governing cell expansion or competence, before the deposition of secondary thickening.

Regulation of *MYB26* Expression

The *ms35* mutation is attributable to a deletion and rearrangement 1288 bp upstream of the translational start of *MYB26*, which results in a severe downregulation of *MYB26* expression and the failure of dehiscence (Steiner-Lange et al., 2003), indicating that the *MYB26* regulatory region extends a considerable distance upstream from the predicted translational start site of the gene (Figure 2). There is a large region (8.124 kb) upstream of *MYB26* before the next annotated gene that does not contain any predicted open reading frames, suggesting that this region may have some role in the regulation of *MYB26* expression. We have identified and characterized two SALK knockout lines that

Figure 1. (continued).

(K) The overexpression (OEx) line OEx4-8, showing less severe phenotypic changes.
(L) Line OEx8-1, showing an extreme dwarf phenotype and only partial fertility.
(M) Nuclear localization of *Pro35S:MYB26:GFP* OEx in root tissues.
(O) Mature silique from a reduced less extreme line, showing deformity attributable to increased secondary thickening.
(N) and **(P)** Overview of mature anthers from the wild type **(N)** and a *MYB26* OEx line **(P)** showing increased lignification in the *MYB26* OEx line.
Bars = 40 μm except in **(G)** to **(J)**, where bars = 20 μm.

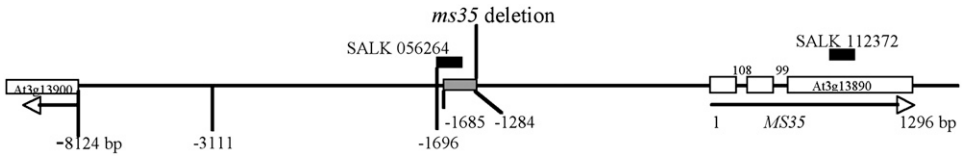

Figure 2. Structure of the *MYB26* Gene.

Map of the *Arabidopsis* At3g13890 region, showing *ms35* deletion and positions of SALK knockout lines. Arrows indicate open reading frames.

contain insertions upstream of *MYB26* (SALK_056264) and within the coding sequence (SALK_112372) (Figure 2). Homozygous knockout lines were generated and screened for the presence of insertions and phenotypic changes; both lines were male-sterile as a result of a lack of secondary thickening in the endothecium and the failure of anther dehiscence. These lines were crossed using pollen from heterozygous *ms35MS35* plants; the resulting progeny from each cross segregated 1:1 for male sterility:fertility (data not shown), indicating that they were allelic to the *ms35* mutation. These knockout lines gave the same phenotype of full sterility attributable to a lack of dehiscence; this was expected in the case of SALK_112372, which lies within the third exon of the *MYB26* transcript. However, the flanking sequence from the SALK_056264 insertion maps to −1696 bp upstream of the translational start (Figure 2). Therefore, the *MYB26* regulatory region may extend as far as −1696 bp upstream from the translational start site, and this region is required for functional expression of the *MYB26* transcript. Bioinformatic analysis of this region has not indicated any definitive motifs that may be responsible for this regulation.

A promoter:β-glucuronidase (GUS) fusion was constructed to localize the expression pattern of *MYB26*; the 3.1-kb region upstream of the transcriptional start site was fused to the GUS reporter gene and transformed into Landsberg *erecta* (L*er*) wild-type plants. Expression of *ProMYB26:GUS* is seen only in floral tissues, with signal observed in the anthers and filaments of the flowers; strong signal is also detected in the style and nectaries of the flowers (Figure 3). Expression is observed in young

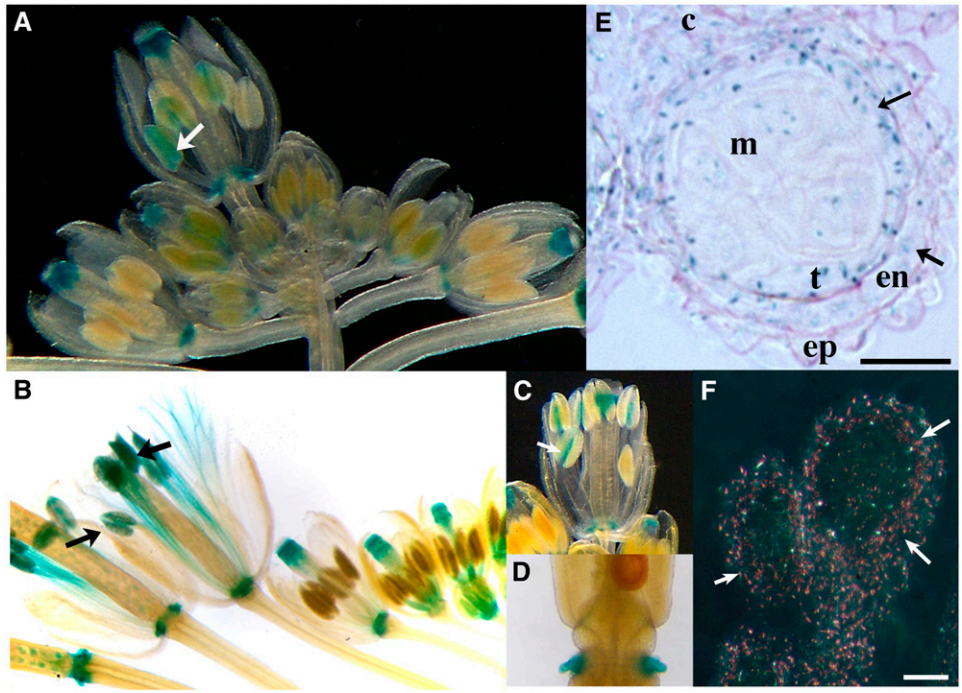

Figure 3. *ProMYB26:GUS* Fusion Expression in *Arabidopsis*.

(A) and **(B)** Inflorescence showing expression of GUS in anthers, filaments, nectaries, and stylar tissues (arrows indicate expression in anther tissue). **(A)** is a dark-field image.

(C) Close-up dark-field image of buds before opening, showing expression in postmeiotic anthers (arrow).

(D) Immature silique showing GUS expression in nectaries.

(E) and **(F)** Sections through anther at pollen mitosis I stage. GUS expression can be seen throughout the anther, particularly in the endothecium and tapetal and connective tissues (arrows). **(E)** is a dark-field image. c, connective tissue; en, endothecium; ep, epidermis; m, microspores; t, tapetum. Bars = 40 μm.

postmeiotic buds through to open flowers (Figures 3A and 3C). Expression is seen at a low level throughout the anther in the tapetum, endothecium, and connective tissues (Figure 3B) and also in the filament tissues (Figures 3A and 3D). Strong GUS staining is also visible in the stylar tissue and nectaries after fertilization, during early seed development (Figure 3E). No signal is detected in vegetative tissues, and no vegetative expression is detected by RT-PCR analysis (data not shown). Although strong GUS staining is seen in the style and nectaries of the ProMYB26:GUS transgenic plants, no significant phenotypic differences associated with an alteration of lignification are observed in these tissues in the *ms35* mutant. A slight reduction in lignification is apparent in *ms35* mutant styles, although lignification is still present and no change in female fertility is detected, because full seed set can be obtained by crossing the male-sterile mutant with pollen from wild-type plants.

The ProMYB26:GUS fusion data were supported by quantitative PCR analysis of *MYB26* expression in staged flowers and anthers (Figure 4). High levels of *MYB26* expression were detected in excised anthers at pollen mitosis I and bicellular stages (Figure 4). Very low levels of expression were detected before pollen mitosis I and in pollen mitosis II, but at a significantly lower level than in pollen mitosis I and bicellular stages. Quantitative RT-PCR analysis of expression was performed in buds, which showed expression in older buds (tricellular pollen stage) and open flowers (see Supplemental Figure 1 online), indicating that this expression corresponds to that seen in ProMYB26:GUS fusion lines in the nectaries and stylar tissue and not to anther expression. Other genes associated with secondary thickening in the anther were also analyzed by quantitative RT-PCR (Figure 4); these show expression at stages after *MYB26*, indicating that they may be regulated by MYB26 or induced by changes in the cell competence linked to MYB26 expression. The timing of *MYB26* expression, before secondary thickening formation, and the altered appearance of the endothecium cells implies that MYB26 may play a regulatory role at an earlier stage associated with the determination of cell expansion or maturation rather than by direct regulation of secondary cell wall thickening.

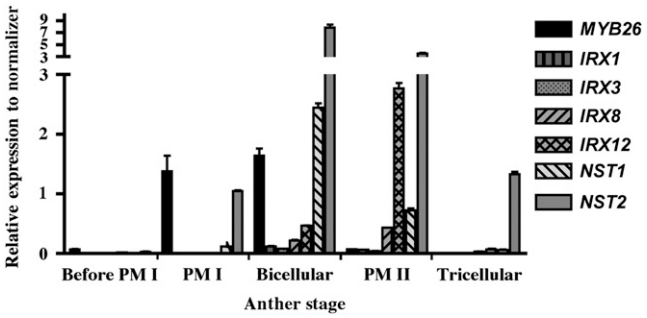

Figure 4. Quantitative RT-PCR Expression in Wild-Type *Arabidopsis* Staged Excised Anthers.

Relative expression levels were determined compared with actin expression and expressed as fold changes relative to actin expression (Stratagene Mx3005P). The data pool consisted of two replicates repeated on at least two separate occasions; error bars show SD of expression changes. PM I, pollen mitosis I; PM II, pollen mitosis II.

Ectopic Lignification Attributable to Overexpression of *MYB26*

The *MYB26* cDNA was cloned from closed buds into the pGWB5 vector (kindly provided by Tsuyoshi Nakagawa, Shimane University) under the control of the cauliflower mosaic virus (CaMV) 35S promoter with a C-terminal green fluorescent protein (GFP) sequence to analyze the effect of ectopic overexpression. This construct was transferred into the wild-type L*er* background and also into heterozygous *ms35 gl1* plants. The *Pro35S:MYB26* transcript partially complemented the *ms35* mutation, causing some restoration of lignification within the endothecium that was sufficient to allow partial anther dehiscence and limited self-fertilization, although complete endothecial thickening did not occur. The fact that endothecial thickening was induced by the Pro35S:MYB26:GFP construct implies that the GFP tag does not affect the function of the MYB26 protein; rather, the CaMV35S promoter was unable to regulate expression in the anther to the same extent as seen with the native *MYB26* promoter.

The pGWB5:*MYB26* construct contains a C-terminal GFP tag, and expression of the GFP could be visualized by confocal microscopy within the nuclei, confirming that MYB26 is localized to the nucleus (Figure 1M), as predicted for its role as a putative transcription factor. Transgenic lines were tested by semiquantitative RT-PCR and quantitative RT-PCR to identify those showing overexpression of *MYB26*. Different levels of expression were seen between the different lines and in the different tissues (see Figures 7 and 8 below), and varying degrees of phenotypic changes were observed between different lines (Figures 1K and 1L). In all cases, the plants overexpressing *MYB26* were reduced in size, which appeared to be attributable to a premature cessation of cell expansion rather than to altered cell number. This variation in size ranged from partially reduced to extremely stunted (Figures 1K and 1L). Changes in the thickening of tissues was evident in many organs of the plant, particularly leaves, petals, and ovules (Figure 5); however, no changes were detected in the root tissues, despite obvious expression in the roots (Figure 1M). Levels of fertility varied, with reduced fertility correlating with levels of ectopic thickening in the reproductive tissues (e.g., OEx8-1 and OEx8-3); very extreme lines were very stunted and completely sterile.

In the floral and reproductive tissues, the ectopic thickening resembled the striated spring-like bands of thickening that are seen in the xylem tissues (Fukuda, 1997) and the anther endothecium (Dawson et al., 1999) (Figures 5A and 5B). However, in the leaf tissues, ectopic lignification of the epidermal cells appeared as a dense net-like pattern that covered the epidermal cell walls (Figures 5C and 5D). The leaves of the plants tended to be distorted and twisted as a consequence of the changes in the thickening of these tissues. In all cases, the thickening was caused by modifications in both secondary thickening and lignification, as indicated by phloroglucinol-HCl and also acridine orange/ethidium bromide staining; however, normal lignification of xylem tissues still occurred in these lines (Figures 5A and 5B).

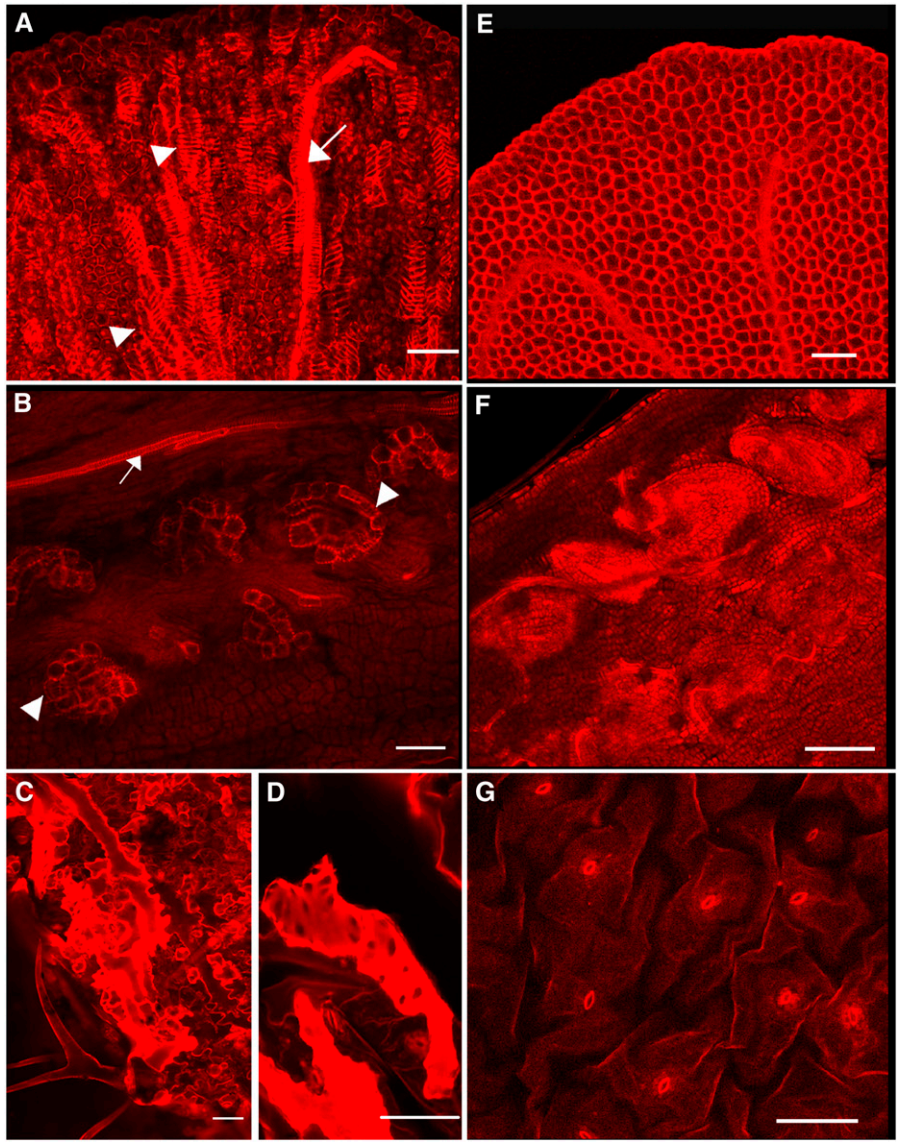

Figure 5. Ectopic Secondary Thickening in Different Tissues in the *Arabidopsis MYB26* Overexpression Lines and Wild-Type L*er*.

Tissues (**[A]** to **[D]**, At *MYB* OEx; **[E]** to **[G]**, wild type) were stained with acridine orange/ethidium bromide and visualized by confocal microscopy (590 nm). Bars = 40 μm.

(A) Petal from a *MYB26* OEx line, showing normal xylem development (arrow) and ectopic spring-like secondary thickening (arrowheads).

(B) Immature ovule from a *MYB26* OEx line, showing normal xylem development (arrow) and ectopic secondary thickening in the epidermal tissues of the ovule (arrowheads).

(C) and **(D)** Leaf tissues from a *MYB26* OEx line, showing net-like ectopic secondary thickening. A more detailed view is shown in **(D)**.

(E) Wild-type petal, showing normal xylem development.

(F) Corresponding wild-type ovule.

(G) Wild-type leaf tissue.

Ectopic secondary thickening and lignification are frequently associated with the epidermal cells (Figures 1 and 5) and involve specific patches of cells within the different tissues rather then all cells within a tissue. This is particularly evident in the epidermal tissues of the anther. In the wild type, secondary thickening occurs in the anther endothecium as bands of striated spring-like thickening (Figures 1A, 1D, 1I, and 1N); this is not seen in the surrounding epidermal cell layers of the anther and is absent in the *ms35* mutant (Figures 1B, 1E, and 1J). However, in the lines overexpressing *MYB26*, patches of epidermal cells within the anther also develop these spring-like secondary thickenings (Figures 1C, 1F, and 1P). These bands of thickening run along the longitudinal axis of the cells; in the resultant architecture, the thickening in the two cell layers, endothecium and epidermis,

runs perpendicular to each other. This means that the bands of thickening run in opposing directions. The result of this is that as the anther dehydrates, opposing forces are generated, rather than the unidirectional force seen in wild-type anthers; therefore, the anther is unable to open fully and is effectively male sterile or of reduced fertility. When released manually, the pollen is viable; however, the extreme lines also show ectopic lignification of the style, ovaries, and ovules, with associated effects on female fertility, and therefore are completely sterile. In some less extreme cases, anther dehiscence and seed set occur, although siliques from these plants are frequently distorted as a consequence of increased thickening (Figure 1O).

Similar patches of ectopic secondary thickening were seen in most of the tissues analyzed except the roots (Figures 1 and 5). The selectivity in cells that developed ectopic thickening was also clearly evident within the ovary, where the ovule wall developed secondary thickening and the other cell layers did not (Figures 5B and 5F). The patchy development of secondary thickening in different cells may be a consequence of levels of *MYB26* expression varying on a cell-to-cell basis, or more likely, given that regulation is by the CaMV35S promoter, a reflection of the competence of those cells to develop secondary thickening.

Ectopic Lignification in Tobacco by Overexpression of *MYB26*

The *Arabidopsis Pro35S:MYB26:GFP* constructs were also transferred into tobacco by *Agrobacterium tumefaciens* transformation of leaf explants and transgenic plants regenerated from transformed callus. The plants showed a similar phenotype to that seen in the *Arabidopsis* OEx lines; they were stunted, bushy, and smaller than wild-type equivalents (Figure 6A) and the leaves, flowers, and stems were much tougher and harder, with increased rigidity. The leaves showed abnormal epidermal cell shape and were contorted as a consequence of increased secondary thickening (Figure 6A). Increased amounts of lignin were associated with the secondary thickening, as indicated by increased levels of phloroglucinol-HCl staining in petals (Figures 6E and 6I). As seen in the *Arabidopsis MYB26* OEx material, no changes in secondary thickening were seen in the root tissues and normal lignification was still observed in other parts of the plants. The most extreme *MYB26* OEx plants were also both male- and female-sterile, with thickening occurring in the ovaries and a failure of dehiscence attributable to an increase in layers of opposing thickening in the anther (Figures 6B to 6D and 6F to 6H). The epidermal tissues of the anther were particularly affected, but the extent of thickening in the other cell layers was also increased greatly (Figures 6C, 6D, 6G, and 6H). Tobacco anthers undergo lignification of both the endothecium and middle cell layers (Sanders et al., 2005), resulting in a double layer of lignified cells surrounding the anther. However, in the wild type, these run in parallel, facilitating anther dehiscence, unlike the situation seen in the OEx lines, in which the epidermal thickening is in the opposite orientation and dehiscence cannot fully occur (Figure 6H).

Petal and anther pigmentation varied in the tobacco OEx lines, with the most severe lines showing a significant reduction of

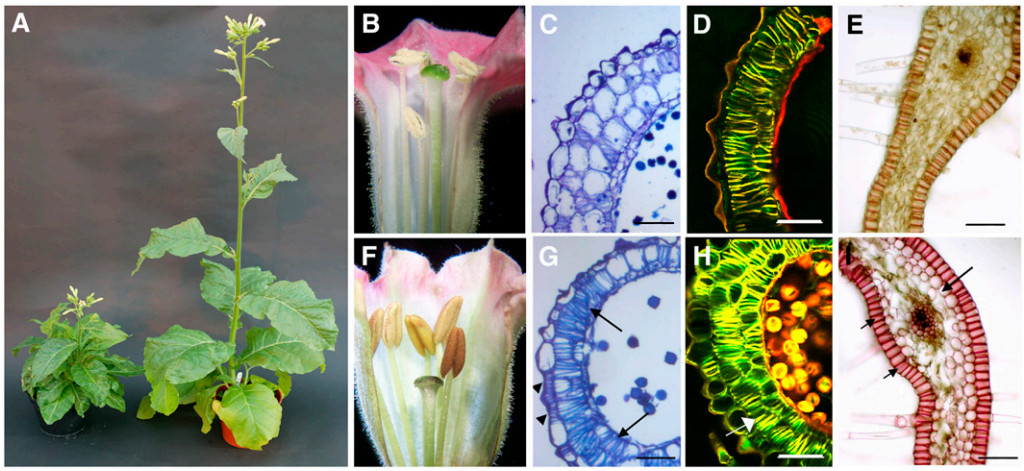

Figure 6. Phenotypic Characterization of Tobacco *Pro35S:MYB26* Overexpression Lines.

(A) A *MYB26* OEx line (left) and a wild-type tobacco plant (right).
(B) to **(E)** Wild-type tobacco.
(F) to **(I)** *MYB26* OEx tobacco.
(B) and **(F)** Open flowers. Anthers in the At *MYB35* OEx line **(F)** fail to fully dehisce, and petal pigmentation is reduced.
(C) and **(G)** Transverse toluidine blue–stained anther sections, showing ectopic secondary thickening in epidermal cells (arrowheads) and increased endothecium and middle cell layer thickenings (arrows) in the OEx lines **(G)**.
(D) and **(H)** Acridine orange/ethidium bromide–stained anthers (merged images; excitation, 520 and 590 nm), showing increased lignification and secondary thickening in the epidermis, endothecium, and middle cell layer of the OEx lines **(H)**.
(E) and **(I)** Phloroglucinol-stained petal sections from the central corolla region. Increased lignification, as indicated by stronger staining, is seen in the epidermis (small arrows) and the ground parenchyma cells of the OEx lines (large arrow).
Bars = 40 μm.

pigmentation in the petals (Figure 6F). This finding suggests that in tobacco overexpression of the pathway regulating secondary thickening also affects flavonoid development; no such changes were evident in the *Arabidopsis* OEx lines.

Expression of Genes Associated with Secondary Thickening in *Arabidopsis* Overexpression Lines

Overexpression of *MYB26* resulted in ectopic secondary thickening and lignification in various organs and cell types within the transgenic lines. The *Arabidopsis* lines carrying the *Pro35S:MYB26:GFP* construct and the *ms35* mutant were analyzed by semiquantitative RT-PCR for alterations in the expression of selected genes linked to secondary thickening (Table 1). These genes fall into a number of distinct classes, including those associated with the regulation of secondary thickening, to key steps in the phenylalanine ammonia-lyase (PAL) pathway, or to cellulose synthesis and secondary thickening (Table 1; see Supplemental Table 1 online). Quantitative RT-PCR analysis was also conducted on a subset of these genes, and the data were analyzed by normalization and relative expression to actin controls using the $2^{-\Delta\Delta}C_T$ method (Livak and Schmittgen, 2001) (Figure 8); the observed expression patterns mimic those seen by semiquantitative RT-PCR analysis.

As expected, *MYB26* expression was decreased in the *ms35* mutant buds and increased in the *MYB26* OEx lines. The OEx lines showed varying levels of expression in the buds and in all cases showed ectopic expression in the leaf tissues, which normally do not express *MYB26* (Figures 7 and 8). No changes in At *MYB32* and At *MYB61* expression were observed in the OEx lines, although the At *MYB32* transcript increased slightly in the *ms35* mutant buds. These MYB transcription factors have been linked to the regulation of phenylpropanoid metabolism and ectopic lignification in *Arabidopsis* (Newman et al., 2004; Preston et al., 2004) respectively. *MYB32* has been shown to be expressed at high levels in the tapetum and to be important for stamen development (Preston et al., 2004; Mandaokar et al., 2006). Therefore, the expression change seen in the *ms35* buds, and the associated lack of change in the OEx lines, are likely to be secondary consequences of changes in anther development and not of direct MYB26 regulation, indicating that MYB26 does not act via a pathway involving either of these transcription factors. We also observed a slight increase in expression of the *ANTHOCYANIDIN SYNTHASE* (*ANS*) gene in *ms35* leaves; no phenotypic changes were seen in the *ms35* mutant leaves, so this is likely to represent secondary changes in the regulation of pathways attributable to the lack of secondary thickening in the anther.

Two NAC domain genes, *NST1* and *NST2*, have been linked to secondary thickening of the anther tissues (Mitsuda et al., 2005), with the overexpression of *NST1* and *NST2* reported to induce ectopic secondary thickening of the anther tissues. Mitsuda et al. (2005) suggested that they may be involved in regulating *MYB26* expression; however, no data were presented to support this. By contrast, our analysis supports the reverse situation, with downregulation of *NST1* and *NST2* in the *ms35* mutant and a corresponding increase of *NST1* and *NST2* expression in the *Pro35S:MYB26* lines (Figures 7 and 8). Analysis of *NST1* and

Table 1. Summary of Semiquantitative RT-PCR of Genes Associated with Lignin and Secondary Thickening

Gene of Interest	AGI Code	Wild-Type Expression		*ms35* Mutant		OEx Lines		Change in *ms35* Mutant	Change in OEx Lines	Putative Role of MYB26
		Leaves	Buds	Leaves	Buds	Leaves	Buds			
NST1	*At2g46770*	*	***	**	**	***	***	Decrease in buds	Increase in leaves	Regulation
NST2	*At3g61910*	–	**	*	*	**	**	Decrease in buds	Increase in leaves	Regulation
MYB32	*At4g34990*	**	**	**	***	**	**	Increase in buds	No change	Secondary effect
MYB61	*At1g09540*	*	**	*	**	*	*	No change	No change	No effect
IRX8	*At5g54690*	*	**	*	*	***	***	Decrease in buds	Increase	Regulation
IRX1	*At4g18780*	*	**	*	**	**	**	Slight decrease	Increase in leaves	Regulation
IRX3	*At5g17420*	*	**	*	**	**	**	No change	Increase	Regulation
IRX5	*At5g44030*	**	***	**	***	**	***	No change	No change	No effect
IRX13	*At5g03170*	*	**	*	**	*	**	No change	No change	No effect
IRX12 (*laccase*)	*At2g38080*	**	**	*	*	**	**	Decrease	Increase	Regulation
XCIP	*At4g35350*	**	**	**	**	**	**	No change	No change	No effect
ANS	*At2g38240*	**	**	***	**	**	**	Increase in leaves	No change	No effect
C4H	*At2g30490*	**	**	**	**	**	**	No change	No change	No effect
DFR	*At1g08200*	***	***	***	***	***	***	No change	No change	No effect
COMT	*At1g67980*	**	**	**	**	***	**	No change	Increase in leaves	Secondary effect
PAL2	*At3g53260*	**	**	**	**	**	**	No change	No change	No effect
IRX4	*At1g15950*	***	***	***	***	***	***	No change	No change	No effect
At3g62160	*At3g62160*	**	**	NT	**	**	**	No change	No change	No effect
AtHB-8	*At4g32880*	**	**	NT	**	**	**	No change	No change	No effect

These data were determined using overexpression lines OEx8-1, OEx8-3, and OEx4-8 (Figure 7). ***, high level of expression; **, moderate level of expression; *, low level of expression; –, no expression detected. AGI, Arabidopsis Genome Initiative; NT, not tested.

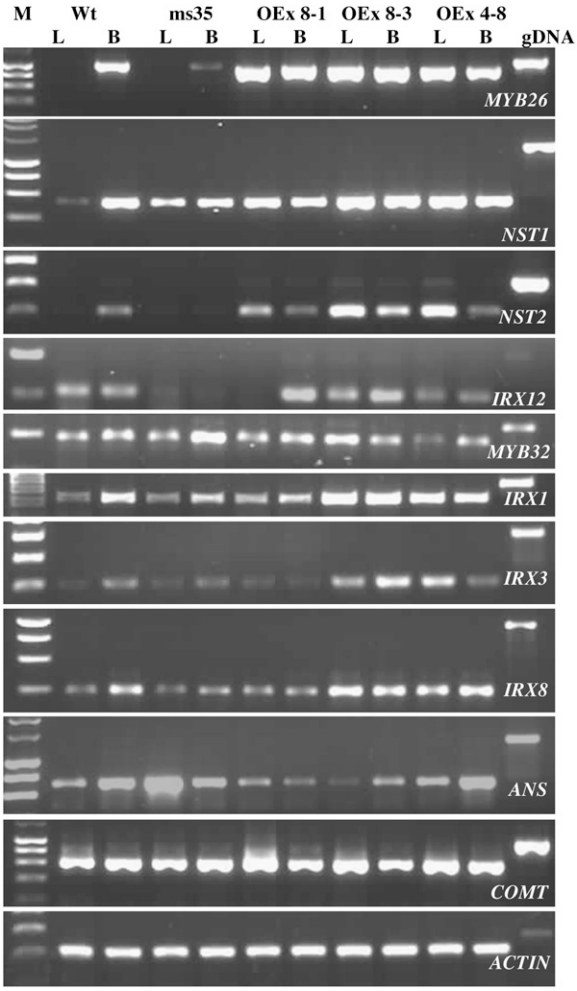

Figure 7. RT-PCR Analysis of Genes Associated with Secondary Thickening in *Arabidopsis* Wild Type, *ms35* Mutant, and *MYB26* Overexpression Lines.

Overexpression lines were OEx8-1, OEx8-3, and OEx4-8 (Table 1; see Supplemental Table 1 online). M, 1-kb marker; Wt, wild type; L, leaf tissue; B, buds; gDNA, genomic DNA control.

NST2 expression in wild-type anthers showed that these genes are expressed after *MYB26* expression at the bicellular and pollen mitosis II stages, although some expression of *NST2* is also evident at the tricellular stage (Figure 4). No significant expression of *NST1* and *NST2* is seen in wild-type leaf tissues, although induction is observed in leaves when *MYB26* is ectopically expressed. In general, the level of *NST1* and *NST2* induction in the different overexpression lines correlates with the level of ectopic *MYB26* expression. In buds, line OEx4-8 shows fewer changes in expression of *MYB26* compared with the wild type and the other overexpressing lines and a corresponding reduced enhancement of *NST1* and *NST2* expression; however, it also shows minimal changes in floral phenotype (Figure 1K). In other lines, such as OEx8-3, the induction of *NST1* and *NST2* is more pronounced, particularly for *NST2* (Figure 8A). The same trends are observed in the leaf tissue, with greater expression of *MYB26* resulting in more *NST1* and *NST2* expression (Figure 8B); this is particularly evident for *NST2* expression. However, the level of change between the two genes, *NST1* and *NST2*, is not always constant. *NST1* and *NST2* have already been shown to act redundantly (Mitsuda et al., 2005); therefore, the level of each is unlikely to be critical at inducing ectopic secondary thickening. This variability of induction indicates that other factors may influence *NST1* and *NST2* expression, particularly because some expression of *NST1* and *NST2* is still seen in the *ms35* mutant, but it may also reflect variation in a cell-specific and temporal manner. *NST1* and *NST2* expression is reduced significantly in the *ms35* mutant; however, a low level of *NST1* and *NST2* expression was still detectable by quantitative PCR, suggesting that there may be additional regulators of their expression. These data suggest that MYB26 acts upstream of these two *NAC* genes, *NST1* and *NST2*, and in turn the formation of thickening in the endothecium. It is unclear at present whether this regulation occurs directly or indirectly; however, analysis of the promoter regions of *NST1* and *NST2* suggests that there are a number of

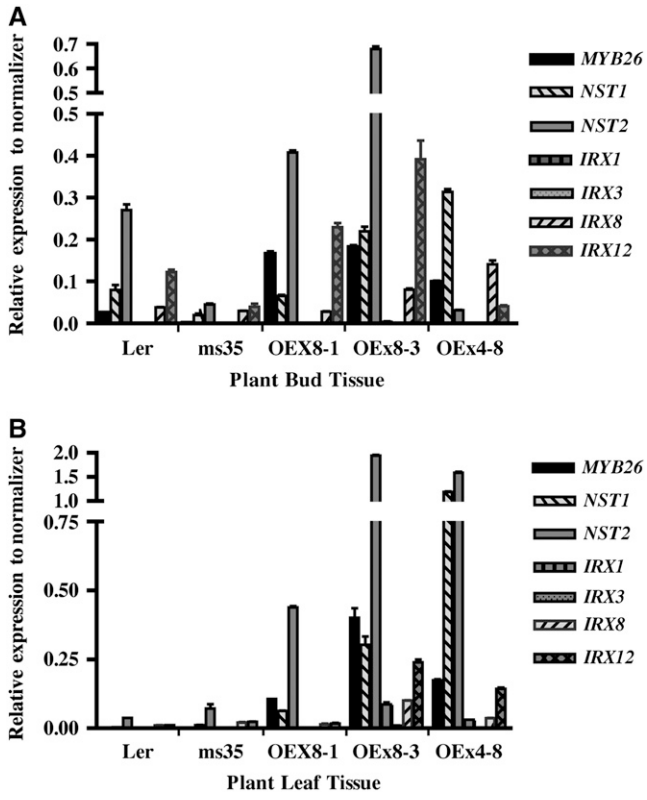

Figure 8. Quantitative RT-PCR of Selected Genes Showing Altered Regulation in the *Arabidopsis MYB26* Overexpression Lines OEx8-1, OEx8-3, and OEx4-8.

Data are shown for bud tissues **(A)** and leaves **(B)**. Relative expression levels were determined compared with actin leaf expression using the $2^{-\Delta\Delta}C_T$ analysis method and are expressed as fold changes relative to actin. The data shown are from two replicates repeated on at least two separate occasions; error bars show SD of expression changes.

putative MYB binding sites in the 5′ upstream regions (TRANSFAC; AthaMap).

A number of genes that have been linked to xylem tracheary element development and secondary cell wall thickening (Table 1; see Supplemental Table 1 online) were also analyzed in the *ms35* mutant and OEx lines. Genes associated with secondary thickening (*IRX1*, *IRX3*, *IRX12* [*laccase*], and *IRX8*) showed changes in expression correlated with levels of *MYB26*, with downregulation in the *ms35* mutant buds and increased expression in the *Pro35S:MYB26* lines (Figures 7 and 8). It has also been demonstrated previously that altered expression of *NST1* results in the overexpression of many of these genes (e.g., *IRX1*, *IRX3*, *IRX4*, *IRX5*, *IRX8*, *IRX13*, and *IRX12*) (Mitsuda et al., 2005). This finding suggests that MYB26 directly, or indirectly possibly via *NST1* and *NST2*, regulates their expression. However, no direct correlation with *MYB26* expression could be seen for a number of key genes from the PAL pathway (Table 1), suggesting that lignification may not be directly regulated by MYB26 or may act via alternative sets of genes involved in lignification. Altered expression levels of *ANS* and caffeic acid *O*-methyltransferase (*COMT*) were seen in the mutant leaves and OEx leaves, respectively (Table 1, Figure 7); however, this did not correspond to changes in the OEx and mutant lines, respectively, suggesting that MYB26 does not directly regulate these genes and that the observed changes are the consequence of secondary changes in flux in the PAL pathway (Figure 9). These data suggest that although the absence of *MYB26* results in a downregulation of many marker genes associated with secondary thickening, no direct changes in genes associated with the PAL pathway are seen. This may be a reflection of subtle changes in gene expression associated with only a limited number of cells within the tissues analyzed, a feature of temporal expression patterns indicating that increased expression of these genes only occurs for a limited period at the onset of the development of secondary thickening, or that the MYB26 does not directly regulate these genes but acts via an additional factor to cause increased secondary thickening.

DISCUSSION

Expression of the *MYB26* Transcript

High-level expression of the *MYB26* transcript appears to require a region extending beyond 1.6 kb upstream of the predicted translational start site, and the absence of putative enhancer sequences in this region results in the lack of dehiscence seen in the *ms35* mutant and the SALK_056264 insertion line. Insertional SALK knockout lines within the *MYB26* open reading frame and the upstream region of *MYB26* have identical phenotypes to the *ms35* mutant. This indicates that although low-level expression is detected in the *ms35* mutant (Figures 7 and 8), the *ms35* mutant serves as a null, with insufficient transcript being produced for endothecial lignification; however, the heterozygote is fully fertile, indicating that a single copy of the *MYB26* transcript is sufficient for normal lignification of the endothecium.

Using a 3.1-kb upstream region from the predicted translational start site fused to a GUS reporter, expression was detected in a number of floral organs, but no vegetative expression was seen. High levels of expression were seen in the style and nectaries of the flowers. Female fertility appears unaffected in the

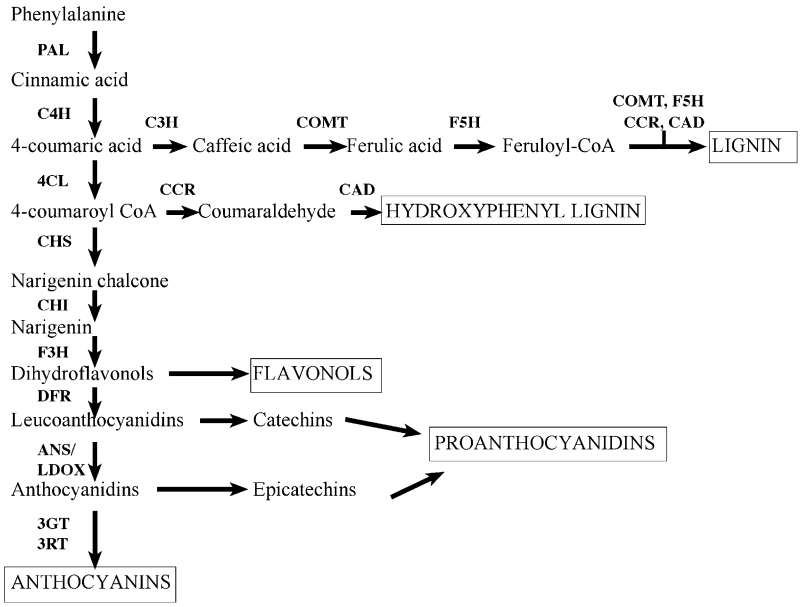

Figure 9. Scheme of the Branch Points in the Phenylpropanoid Pathway.

This figure is adapted from Deluc et al. (2006). ANS/LDOX, anthocyanidin synthase; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamyl-CoA reductase; C3H, 4-coumarate-3-hydroxylase; C4H, cinnamate 4-hydoxylase; CHI, chalcone isomerase; CHS, chalcone synthase; 4CL, 4-coumarate-CoA ligase; COMT, caffeic acid *O*-methyltransferase; DFR, dihydroflavonol 4-reductase; F3H, flavonoid 3-hydroxylase; F5H, ferulate 5-hydroxylase; 3GT, anthocyanidin 3-glucosyltransferase; PAL, phenylalanine ammonia-lyase; 3RT, anthocyanidin-3-glucoside rhamnosyl transferase.

ms35 mutant, indicating that the style is fully functional. Lignin development, although reduced, still occurs within the style, implying that there may be redundant genes regulating secondary thickening within the style, so that secondary thickening still occurs in the absence of *MYB26*, albeit at a slightly reduced level. Nectaries have a role in the production of nectar for insect attraction to facilitate pollination; however, because *Arabidopsis* is predominantly self-fertile, the nectaries may not have a functional role in pollination and fertilization. Expression of the *MYB26* transcript is detected in these tissues, although no significant differences in secondary thickening were observed, which may either reflect a lack of functional MYB26 protein in these tissues or indicate a redundancy of gene regulation. Expression of a number of genes linked to PAL metabolism (Thoma et al., 1994) and dehiscence (Rajani and Sundaresan, 2001) has also been observed in *Arabidopsis* nectaries, although alterations in phenotype have not been linked to these expression patterns. Expression, as indicated by GUS promoter fusion data and quantitative RT-PCR, is seen in the anthers, from the start of pollen mitosis I through to bicellular pollen, although expression in the nectaries and style is maintained beyond these stages. This pattern of anther expression coincides with the differentiation of cell types within the anther and the expansion of the endothecial cells immediately before the initiation of secondary thickening in the endothecium and vascular tissues in the filament. Immediately after *MYB26* expression, other genes directly linked to secondary thickening are expressed, implying that *MYB26* acts upstream of secondary thickening via the regulation of genes such as the NAC domain genes *NST1* and *NST2* and may direct cell expansion and the change of cell competence toward secondary thickening, rather than directly inducing the expression of genes in the secondary thickening pathways.

Induction of Secondary Thickening

Ectopic secondary thickening was induced in both *Arabidopsis* and tobacco plants by overexpression of the *MYB26* transcript. In general, the severity of the phenotype correlated with the level of *MYB26* transcript, suggesting that the amount of MYB26 protein is linked to the extent of phenotypic changes. Increased secondary thickening and lignification were seen in these lines.

A number of MYB factors have been linked to PAL metabolism and secondary thickening. *Eucalyptus grandis* Eg MYB2, which has homology with *Arabidopsis* At MYB83 and *Populus tremuloides* Pt MYB4, has been shown to regulate expression by binding to MYB binding sites in the promoters of cinnamoyl-CoA reductase and cinnamyl alcohol dehydrogenase genes (Goicoechea et al., 2005). Tobacco plants overexpressing *Eucalyptus MYB2* showed increased secondary cell wall thickness and altered lignification profiles, indicating that *Eucalyptus MYB2* has a role in the coordinated control of genes involved in the monolignol-specific pathway; however, other core PAL genes remain unaffected (Goicoechea et al., 2005). *Arabidopsis MYB61* has also been linked to the ectopic lignification and dark photomorphogenic phenotype of *deetiolated3* (Newman et al., 2004). *Arabidopsis* MYB32 has also been shown to regulate genes in the PAL pathway, with downregulation of the *MYB32* gene increasing transcript levels of the *COMT* gene and reducing levels of the DIHYDROFLAVONOL 4-REDUCTASE (*DFR*) and *ANS* genes (Preston et al., 2004). Despite anther expression, the pattern of *MYB32* expression is very different from that of *MYB26*; *MYB32* is expressed in all major organs, but with a high level of expression in the tapetum and the stigma (Preston et al., 2004), whereas expression of *MYB26* occurs specifically within the endothecium tissues, style, and nectaries. *MYB32* does not appear to affect the dehiscence process but is thought to alter pollen wall formation by affecting wall development, resulting in collapsed inviable pollen (Preston et al., 2004). No change of expression was seen in either of these two *MYB* factors in the *MYB26* overexpression lines, indicating that MYB26 is upstream of, or does not induce, secondary thickening via *MYB32* or *MYB61*.

IRX1, *IRX3*, *IRX8*, and *IRX12* expression appears to be correlated directly with levels of *MYB26*, suggesting direct or indirect regulation by MYB26. Similar increased expression of these genes was also observed in the *Pro35S:NST1*-overexpressing lines (Mitsuda et al., 2005). *IRX1*, *IRX3*, and *IRX5* have been shown to be coexpressed in secondary cell walls and to encode essential components of the cellulose-synthesizing complex (Taylor et al., 1999, 2003). Transcriptomic analysis has shown that *IRX1* and *IRX3* exhibited similar expression patterns during hypocotyl and stem development, and their expression pattern has been linked by expression profiling to a number of genes associated with secondary thickening, including *IRX8* (a glucosyl transferase involved in pectin synthesis) and *IRX12* (*laccase*) (Brown et al., 2005). *IRX3* and *laccase* (*IRX12)* have both been shown to be regulated by *VND7* during transdifferentiation of xylem vessel elements (Kubo, 2005). The altered expression of these genes in the *Pro35S:MYB26:GUS* lines suggests that MYB26 directly or indirectly regulates a group of key genes associated with secondary wall formation, possibly via regulation of *NST1* and *NST2* expression.

Lignin is derived from the dehydrogenative polymerization of hydroxycinnamyl alcohols (monolignols) and other phenylpropanoids (Raes et al., 2003). Biosynthesis of monolignols requires enzymes of the phenylpropanoid (PAL) pathway (Figure 9) using derivatives of Phe. Lignin is a major component of secondary thickening of cell walls, resulting in the development of large amounts of biomass and providing physical strength for plants combined with the physical forces required for dehiscence, pod shatter, etc. Many mutants have been identified in biosynthetic pathways of lignin, but only recently have genes been identified associated with the regulation of this pathway (see Supplemental Table 1 online). Lignification is usually seen in sclerified cells and requires the coordinated expression of a number of genes from the PAL pathway and those associated with the polymerization of monolignols. Therefore, there is a need for a central regulatory system for the induction of lignin development, which then switches on the other components required for lignification. MYB proteins have been linked to such a regulatory role; two *Antirrhinum* MYB transcription factors have been shown to regulate the expression of genes in the PAL pathway (Tamagnone et al., 1998). *MYB46* is a predicted ortholog of pine (*Pinus*) *MYB4*, a positive regulator of the lignification of xylem cells and phloem fibers in loblolly pine (*Pinus taeda*) (Patzlaff et al., 2003), and MYB52, a predicted ortholog of *Populus tremula* × *P. tremuloides MYB21a*, is a repressor of caffeoyl-CoA 3-*O*-methyltransferase

expression (Karpinska et al., 2004). Expression of NAC and MYB factors has been associated with the transcriptome of xylem and phloem development in *Arabidopsis* root hypocotyls (Zhao et al., 2005). Conserved activator elements, which are critical for the regulation of expression, have been identified in promoters of PAL pathway genes (Neustaedter et al., 1999) and are similar to the motifs recognized by MYB proteins. In the case of the PAL promoter, these have been shown to bind and regulate GUS reporter expression (Sablowski et al., 1994). No changes were evident in pigment formation and flavonoid gene expression in the *Arabidopsis* OEx material, although changes in pigmentation in petal and anther were seen in the tobacco OEx lines, suggesting that there may be subtle alterations in the regulation of components of the PAL pathway in tobacco compared with *Arabidopsis*. Similar effects were observed in grapevine (*Vitis vinifera*) by overexpression of Vv *MYB5a*, which resulted in an increase in pigment formation in stamens, enhanced anthocyanin biosynthesis, and increased expression of genes in the flavonoid biosynthetic pathway, such as *CHALCONE SYNTHASE*, *CHALCONE ISOMERASE*, *FLAVANONE 3-HYDROXYLASE*, and *DFR* (del Rio et al., 2004).

The fact that ectopic production of lignin is seen in the *MYB26* OEx *Arabidopsis* and tobacco lines suggests that MYB26 has a key role in the regulation of genes in the PAL and monolignol polymerization pathways and that the regulation of this pathway appears to be evolutionarily conserved between tobacco and *Arabidopsis*. However, no direct increase in gene expression of a number of specific genes from the PAL pathway was seen in OEx *MYB26* lines, although changes in *IRX12* (*laccase*) expression were seen. This finding suggests that the regulation of PAL pathway genes is either extremely transient and low-level or that the role of MYB26 in lignification may be indirect or via alternative members of these pathways, because there is a high level of redundancy in the PAL pathway. Our data indicate that MYB26 acts by inducing the expression of the NAC domain genes *NST1* and *NST2*, which were previously linked to expression changes associated with secondary thickening and lignification (Mitsuda et al., 2005). The NAC transcription family has been linked to maintaining tissue boundaries, regulating the control of growth from cell division to expansion, and other developmental processes such as senescence (Zhao et al., 2005). NST1 and NST2 appear to act redundantly in the regulation of secondary cell walls in the anther (Mitsuda et al., 2005), and a similar phenotype to that seen in the *Pro35S:MYB26* lines of ectopic secondary thickening was observed when *NST1* or *NST2* was expressed using the CaMV35S promoter. It was suggested previously that *NST1* and/or *NST2* may regulate the expression of *MYB26* (Mitsuda et al., 2005); however, this contradicts the changes of *NST1* and *NST2* expression seen in our data. *NST1* and *NST2* exhibit different but overlapping expression patterns: *NST1* is seen in many tissues of the plant, including inflorescence stems, mid ribs of leaves and anthers, filaments, stamens, and carpels, whereas *NST2* shows a much more specific pattern, with expression in anther walls and pollen grains (Mitsuda et al., 2005). This is in contrast with the observations of *MYB26* expression, which is seen only in the reproductive tissues and principally in the filament, anther, nectaries, and style. Therefore, *MYB26* may play a role in establishing cell competence and determining which cells are capable of undergoing secondary thickening by regulating *NST1* and *NST2* expression in these tissues.

The induction of ectopic secondary thickening does not occur in all cells, but it is evident predominantly in epidermal cells of both *Arabidopsis* and tobacco *MYB26* OEx lines. The epidermis has been shown to be a highly metabolically active cell type; for example, in *Catharanthus roseus*, concomitant expression of genes from at least four separate metabolic pathways, including those from the PAL pathway, was seen in the epidermal tissues (Mahroug et al., 2006). The development of thickening predominantly in the epidermis of the OEx lines may reflect the competence of the epidermal tissue for such metabolic activity. However, not all cells undergo thickening; some were visible as ectopic islands among cells showing normal development. This sporadic thickening has also been described in other reports of ectopic secondary thickening (Mitsuda et al., 2005). This may be a consequence of differences in the cell-specific expression of the transgene, but more likely it reflects a competence for secondary thickening in those cells that is associated either with the cessation of cell growth or with the ability to respond to signals required for secondary thickening.

This work has shown that the processes of secondary thickening are conserved between *Arabidopsis* and tobacco and that overexpression of *MYB26* results in ectopic induction of secondary thickening and lignification. This is seen in selected cells within a variety of tissues, suggesting that some aspect of competence is required for the induction of thickening. These data suggest that MYB26 may either regulate genes associated with secondary thickening in the endothecium or else function in specifying cell competence and determining which cells undergo secondary thickening. MYB26 appears to act by regulating the expression of *NST1* and *NST2*, which may directly or indirectly affect the levels of genes associated with the development of secondary thickening. The ability to regulate secondary thickening in a conserved manner has significant commercial applications for the wood and paper industries.

METHODS

Plant Growth Conditions

Seeds of *Arabidopsis thaliana* var L*er* and the *ms35gl* mutant were sown onto a compost mix of Levington M3:vermiculite (3:1) and grown in a glasshouse at 21/17°C (day/night) with a 22/2-h photoperiod as described previously (Dawson et al., 1999). The T-DNA insertion lines SALK_056264 and SALK_112372 were generated by the Salk Institute Genomic Analysis Laboratory and obtained from the Nottingham Arabidopsis Stock Centre (NASC). Both T-DNA insertion lines and wild-type (ecotype Columbia) control plants were grown as described previously. Plants were genoptyped by PCR using SALK_LBb1 (5′-GCGTGGACCGCTTGCTGCAACT-3′), SALK_112372_RP (5′-CATTGAGCTTCACAGCATTCTTGG-3′), and SALK_112372_LP (5′-GTCCACAAGAGATTGGCGACG-3′) primers for SALK_112372 and SALK_LBb1 (5′-GCGTGGACCGCTTGCTGCAACT-3′), SALK_056264_LP (5′-CCGCGGGTTAAAATCTATTATGTGA-3′), and SALK_056264_RP (5′-TCGAACGTACGTATGTAGAGTCCT-3′) primers for SALK_056264.

Transformation of Tobacco

Tobacco (*Nicotiana tabacum*) seeds were surface-sterilized and sown onto MSR3 (Liu, 2005) medium under conditions of 16 h of daylight at

25°C followed by 8 h of dark at 18°C. Fully expanded, 3- to 4-week-old tobacco leaves were used as explants. Transgenic tobacco plants were generated by *Agrobacterium tumefaciens*–mediated leaf slice transformation (Liu, 2005) and selected for kanamycin resistance. Regenerated transgenic plants were moved onto compost and grown in the glasshouse as described for *Arabidopsis*.

MYB26 Promoter::GUS Construct

A 3.127-kb region upstream of the *MYB26* gene was amplified by PCR (MS35-Xho1-3540L, 5′-GCCTCGAGAAGGGAAGCCTCCGACAGAA-3′; MS35R-AVRII-14R, 5′-AGCCTAGGCTAGATCTCTATCGCTCTCTTAG-TCT-3′), digested with *Xho*I/*Avr*II, and cloned upstream between the *Xho*I and *Xba*I sites of the *uidA* gene of the MOG402-based binary vector p*MOGMS1:GUS* (Wilson et al., 2001), replacing the MS1 promoter to create p*MOGMYB26:GUS*. The construct was then transferred into *Agrobacterium* (C58 pGV3850) by electroporation (Sambrook et al., 1989) and transformed into *Arabidopsis* (L*er*) plants by floral dipping (Clough and Bent, 1998). β-Glucuronidase activity was visualized by staining the inflorescences overnight in 5-bromo-4-chloro-3-indolyl-β-glucuronic acid solution (Willemsen et al., 1998).

Overexpressing Lines

The 1135-bp *MYB26* coding region was amplified by PCR (FGWMS35cDNA, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCA-TGGGTCATCACTCATGCTG-3′; RGWMS35cDNA, 5′-GGGGACCACTT-TGTACAAGAAAGCTGGGTCAGTTATGACGTACTGTCCACAAGAGAT-3′) from L*er* bud cDNA, cloned by recombination into Gateway pDONR201 entry vector (Invitrogen), and transferred into pGWB5 (Karimi et al., 2002) under the control of the CaMV35S promoter. The construct was then transformed into *Arabidopsis* and tobacco plants as described above. Plants were selected on Murashige and Skoog plates containing 50 mg/mL kanamycin and screened for the presence of the transgene by PCR.

Expression Analysis

RNA was isolated from buds and leaves (RNeasy; Qiagen), and cDNA was prepared using 5 μg of total RNA in a 20-μL reaction (SuperScript II reverse transcriptase; Invitrogen). This was used for RT-PCR analysis using 0.5 μL of cDNA template and the appropriate primer pairs (see Supplemental Table 1 online) for 30 cycles at 94°C for 30s, 58°C for 30s, and 72°C for 1 min. RT-PCR results were normalized using *Arabidopsis* actin-2 controls (Wilson et al., 2001).

Quantitative RT-PCR

Quantitative RT-PCR analyses were performed using the Mx3005P multiplex quantitative PCR system (Stratagene). Reactions were set up using the Brilliant SYBR Green QPCR Master Mix (Stratagene) in a final volume of 25 μL containing 0.5 μL of cDNA and 0.5 μL of the appropriate primers (see Supplemental Table 1 online). PCR cycling conditions for amplification were 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min. All samples were run at least in duplicate. Data acquisition and analyses were performed using Mx3005TM multiplex quantitative PCR system software. Relative expression levels were determined compared with actin leaf expression using the $2^{-\Delta\Delta}C_T$ analysis method (Livak and Schmittgen, 2001).

Microscopy

For analysis of lignin, fresh samples or tissues were stained with phloroglucinol-HCl (Ruzin, 1999) and were observed with a light microscope (Nikon). For confocal microscopy (TCS SP2; Leica), a modified ethidium bromide/acridine orange stain was used (A.M. Patten, personal communication); the ethidium bromide stains lignified cells (red fluorescence; excitation, 590 nm) and the acridine orange stains nonlignified walls (green fluorescence; excitation, 520 nm). Fresh tissues were washed (1× PBS and 2% [v/v] Tween 20 for 10 min, then 1× PBS), stained with 0.01% (w/v) acridine orange (1 h, room temperature), washed (1× PBS), and then stained with 0.00005% (w/v) ethidium bromide (1 h, room temperature).

Plant material was also fixed and embedded in paraffin, and sections were stained with toluidine blue, as described previously (Vizcay-Barrena and Wilson, 2006). A minimum of 10 independent transformants were analyzed.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the key genes mentioned in this article are as follows: *MYB26* (At3g13890), *NST1* (At2g46770), *NST2* (At3g61910), *IRX1* (At4g18780), *IRX3* (At5g17420), *IRX5* (At5g44030), *IRX8* (At5g54690), and *IRX12* (At2g38080); others are listed in Table 1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Primer Sequences for Genes Associated with Lignin and Secondary Thickening.

Supplemental Figure 1. Quantitative RT-PCR Expression in Wild-Type *Arabidopsis* Flowers and Buds.

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