MYB75 Functions in Regulation of Secondary Cell Wall Formation in the Arabidopsis Inflorescence Stem[1][W]

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Deposition of lignified secondary cell walls in plants involves a major commitment of carbon skeletons in both the form of polysaccharides and phenylpropanoid constituents. This process is spatially and temporally regulated by transcription factors, including a number of MYB family transcription factors. MYB75, also called *PRODUCTION OF ANTHOCYANIN PIGMENT1*, is a known regulator of the anthocyanin branch of the phenylpropanoid pathway in Arabidopsis (*Arabidopsis thaliana*), but how this regulation might impact other aspects of carbon metabolism is unclear. We established that a loss-of-function mutation in MYB75 (*myb75-1*) results in increased cell wall thickness in xylary and interfascicular fibers within the inflorescence stem. The total lignin content and S/G ratio of the lignin monomers were also affected. Transcript profiles from the *myb75-1* inflorescence stem revealed marked up-regulation in the expression of a suite of genes associated with lignin biosynthesis and cellulose deposition, as well as cell wall modifying proteins and genes involved in photosynthesis and carbon assimilation. These patterns suggest that MYB75 acts as a repressor of the lignin branch of the phenylpropanoid pathway. Since MYB75 physically interacts with another secondary cell wall regulator, the KNOX transcription factor KNAT7, these regulatory proteins may form functional complexes that contribute to the regulation of secondary cell wall deposition in the Arabidopsis inflorescence stem and that integrate the metabolic flux through the lignin, flavonoid, and polysaccharide pathways.

The allocation of carbon to different metabolic pathways in plants is a central feature of growth and development in plants (Bloom et al., 1985; Smith and Stitt, 2007). However, the molecular underpinnings regulating the sensing and signaling system(s) that are anticipated to link carbon assimilation to particular metabolic pathways have yet to be identified. Plant secondary cell walls represent a major carbon sink in plants (Brown et al., 2005; Pauly and Keegstra, 2008), and many proteins catalyzing deposition of secondary cell wall polysaccharides and lignin have been characterized (Zhong and Ye, 2007). For example, the synthesis of cellulose at the plasma membrane during both primary and secondary cell wall synthesis has been shown to be dependent on distinct Cellulose Synthase A (CESA) proteins. The deposition of cellulose in the secondary cell wall involves CESA4, 7, and 8 in Arabidopsis (*Arabidopsis thaliana*; Somerville et al., 2004; Brown et al., 2005; Persson et al., 2005), while primary cell wall cellulose formation is orchestrated by CESA3, 6, and 9 (Joshi and Mansfield, 2007). Additional polysaccharide components are synthesized by enzymes involved in hemicellulose production, primarily of the glycosyltransferase families (Persson et al., 2007; Brown et al., 2009). Secondary cell wall lignin biosynthesis requires the activity of both core phenylpropanoid pathway enzymes, such as L-Phe ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H), as well as enzymes more directly engaged with lignin biosynthesis, including caffeoyl-CoA *O*-methyltransferase (CCoAOMT; Do et al., 2007), ferulate 5-hydroxylase (F5H; Meyer et al., 1998), cinnamoyl-CoA reductase 1 (CCR1; Mir Derikvand et al., 2008), and cinnamoyl alcohol dehydrogenase (CAD; Sibout et al., 2005). The regulation of the corresponding genes is, in part, facilitated by a number of transcription factors that have been shown to regulate secondary cell wall formation in Arabidopsis and other plants and include members of the NAC domain (Kubo et al., 2005) and MYB families of transcription factors (Zhong and Ye, 2007, 2009).

Lignin biosynthesis represents a major terminal product of phenylpropanoid metabolism, a multibranched system of reactions that converts the carbon skeleton of L-Phe into a wide variety of phenolic plant metabolites. Another major branch of phenylpropanoid metabolism generates flavonoids, a diverse group of phenolics that includes flavonols, isoflavonoids, leucoanthocyanidins (tannins), and anthocyanins. Various transcription factors, including a number of MYB family proteins, have been shown to directly or indirectly regulate the activity of genes encoding enzymes involved in specific branches of the phenylpropanoid pathway (Davies and Schwinn, 2003; Dubos et al., 2005;

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Gonzalez et al., 2008; Zhong and Ye, 2009), but less is known about the transcriptional regulation of carbon partitioning across different branches of phenylpropanoid metabolism by individual transcription factors.

MYB75 (At1g56650), also known as *PRODUCTION OF ANTHOCYANIN PIGMENT1* (*PAP1*), was earlier identified as a positive regulator of anthocyanin biosynthesis in Arabidopsis, based on the strong accumulation of anthocyanins in activation-tagged seedlings overexpressing the *MYB75/PAP1* gene (Borevitz et al., 2000; Pourtau et al., 2006; Gonzalez et al., 2008). In addition to this impact of PAP1 overexpression on anthocyanin production, the activity of *PAP1/MYB75* has been found to influence senescence (Bernhardt et al., 2003), Suc signaling (Teng et al., 2005), and lignin deposition in Arabidopsis (Borevitz et al., 2000). A loss-of-function *MYB75* allele (*myb75-1*) harbors mutations inside the DNA-binding domain of the encoded protein (Teng et al., 2005), and *myb75-1* plants show only a very weak anthocyanin accumulation response to elevated levels of Suc. The expression of the MYB75 gene is also responsive to a number of abiotic factors, including nitrogen deficiency, phosphate starvation, high temperature, and high light intensity (Misson et al., 2005; Tohge et al., 2005a, 2005b; Vanderauwera et al., 2005; Lea et al., 2007; Morcuende et al., 2007; Muller et al., 2007; Rowan et al., 2009), and this spectrum of metabolic and environmental sensitivities suggests that, in addition to modulating anthocyanin biosynthesis, MYB75 might play a more general role in regulating cellular metabolism.

Since the secondary cell wall represents one of the main carbon sinks in plants and its formation requires coordination of metabolic fluxes through both polysaccharide and phenylpropanoid biosynthetic pathways, we postulated that transcription factors, such as MYB75, could be involved in the allocation of photosynthetic carbon between these two dissimilar, yet highly important, end-products. The Arabidopsis inflorescence stem has a high developmental commitment to secondary wall formation, which makes this tissue particularly well suited to study the regulation of secondary cell wall by specific transcription factors. We show here that while *MYB75* overexpression results in general up-regulation of anthocyanin accumulation, as previously reported (Borevitz et al., 2000), a *myb75* loss-of-function mutant displays an overall increase in secondary cell wall formation in the inflorescence stem, accompanied by elevated expression of genes encoding enzymes integral to the biosynthesis of lignin and secondary cell wall polysaccharides. This suggests that MYB75 may be acting as a repressor of the lignin branch of the phenylpropanoid pathway.

RESULTS

MYB75 Expression in Wild-Type and Gain- and Loss-of-Function Mutants

To study the role of MYB75 in regulating cell wall biosynthesis and phenylpropanoid metabolism, we compared an activation-tagged *MYB75* gain-of-function mutant (Supplemental Fig. S1A; *pap1-D*), referred to here as *MYB75(o/x)*, with a loss-of-function transposon-tagged *Ds* insertion mutant (*myb75-1*; Supplemental Fig. S1A). Plants homozygous for both *MYB75(o/x)* and *myb75-1* alleles were identified by PCR-aided genotyping, and the abundance of *MYB75* transcripts was assessed using quantitative real-time PCR (qRT-PCR). As expected, *MYB75* transcript abundance was higher in the overexpression line than in wild-type plants, while negligible *MYB75* expression was detected in the loss-of-function mutant (Supplemental Fig. S1B). *MYB75(o/x)* seedlings showed elevated levels of anthocyanin accumulation under normal growth conditions, as reported earlier (Borevitz et al., 2000), while the anthocyanin content was slightly reduced from wild-type levels in *myb75-1* seedlings (Supplemental Fig. S1C), a pattern that is consistent with MYB75 acting as a positive regulator of this branch of the phenylpropanoid pathway. No visible difference in growth or inflorescence stem morphology was observed when the mutants were compared with their corresponding wild-type controls (Supplemental Fig. S1D) and grown under normal growth conditions.

When *MYB75* expression was assessed in various tissues of 6-week-old plants by qRT-PCR, transcript levels were found to be highest in the lower part of the inflorescence stem (Fig. 1A). Lower levels of *MYB75* transcripts could be detected in flowers, leaves, and siliques, but none could be detected in roots (Fig. 1A).

To obtain a spatially and developmentally better resolved picture of the expression of *MYB75*, transgenic Arabidopsis plants containing a *MYB75pro*::*GUS* transgene were examined using histochemical staining for GUS activity (Fig. 1B). It had been previously reported that *GUS* expression is seen in most parts of *MYB75pro*::*GUS* seedlings (Gonzalez et al., 2008), but in 6-week-old plants, we found that GUS activity was primarily localized in the vasculature of leaves and flowers (Fig. 1B, a and b) and in the epidermis of siliques (Fig. 1B, c), but not in roots (data not shown), a pattern consistent with the qRT-PCR data. Within the lower portion of the inflorescence stem, where the highest levels of *MYB75* transcript had been detected, GUS activity was observed specifically in the cortex, vascular bundles, and fibers (Fig. 1B, d).

MYB75 Is Nuclear Localized and Acts as a Transcriptional Activator

We used an Arabidopsis protoplast transient expression system to assay the subcellular localization of a MYB75-yellow fluorescent protein (YFP) fusion, which was found to accumulate in the nucleus (Fig. 2A). A protoplast transfection system (Wang et al., 2007, 2008) was also used to assess the transcriptional repression or activation activity of *MYB75*. Cotransfection of a *GAL4*:*GUS* reporter construct with an effector construct containing the *MYB75* open reading frame fused to the GAL4 DNA-binding domain (GD;

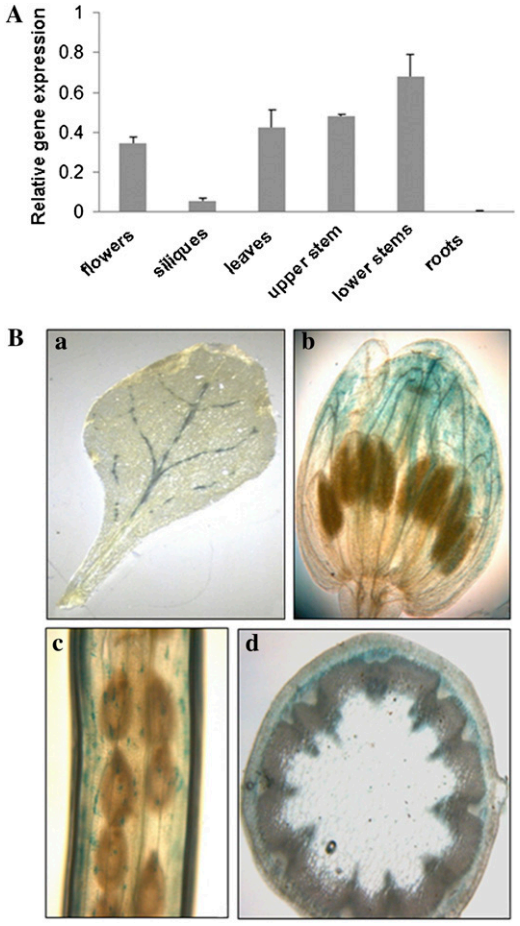


Figure 1. Expression pattern of the *MYB75* in mature Arabidopsis plant and stems. A, qPCR analysis showing the predominant expression of *MYB75* in stems. The expression level of *MYB75* is relative to actin and is expressed in arbitrary units. Error bars represent SE of three biological replicates. B, GUS expression pattern in different organs of mature *MYB75*::*GUS* plants: in leaf midrib (a), in the epidermal cells of the flower (b), in the epidermis of the silique (c), and in cortex and in vascular bundles in the cross section of lower inflorescence stem (d).

Fig. 2B) revealed that MYB75 could weakly activate expression of the *GUS* reporter gene when recruited to the promoter region of the reporter gene by GD (Fig. 2B). To test the possibility that MYB75 might also be able to act as a transcriptional repressor, we coexpressed a construct containing the *GUS* gene driven by the 35S promoter of *Cauliflower mosaic virus* supplemented with both LexA and Gal4 DNA binding sites. When cotransfected with both the MYB75-GD effector construct and the transactivator LD-VP16, the MYB75-GD gene product failed to suppress activation of the GUS reporter by LD-VP16. As a positive control, we also coexpressed the target reporter with a KNAT7-GD construct that had been previously shown function as a repressor (E. Li, S. Wang, J.-G. Chen, and C.J. Douglas, unpublished data). As expected, *KNAT7* expression strongly reduced *GUS* expression from the 35S/LexA/Gal4 *promoter*::*GUS* reporter (data not shown). Taken together, these data suggest that MYB75 may act as a weak transcriptional activator but not as a repressor.

Loss of *MYB75* Function Affects Secondary Cell Wall Structure and Composition

The RIKEN line pst16228 (*myb75-1* loss of function) has a transposon inserted in the third exon of *MYB75/PAP1* (Supplemental Fig. S1), and this Ds insertion event is tightly linked to the phenotype of *myb75-1* (Teng et al., 2005). No other loss-of-function alleles appear to be available, but a MYB75/PAP1-RNA interference line has been reported to display an anthocyanin-deficient phenotype similar to that of pst16228 seedlings (Gonzalez et al., 2008), confirming that this pigment phenotype is due to loss of *MYB75/PAP1* function.

When the basal portion of the inflorescence stem in *myb75-1* plants was examined in Toluidine Blue-stained cross sections (Fig. 3A), and by transmission electron microscopy (TEM; Fig. 3B), the secondary cell walls of the interfascicular fibers appeared to be thicker, compared with wild-type plants, while no change in vessel wall thickness or cell morphology was apparent (Fig. 3, A and B). Measurements taken

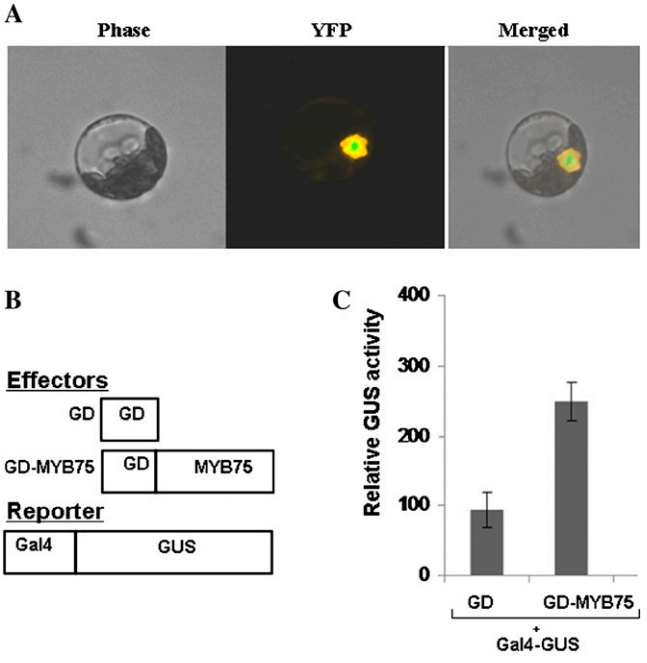
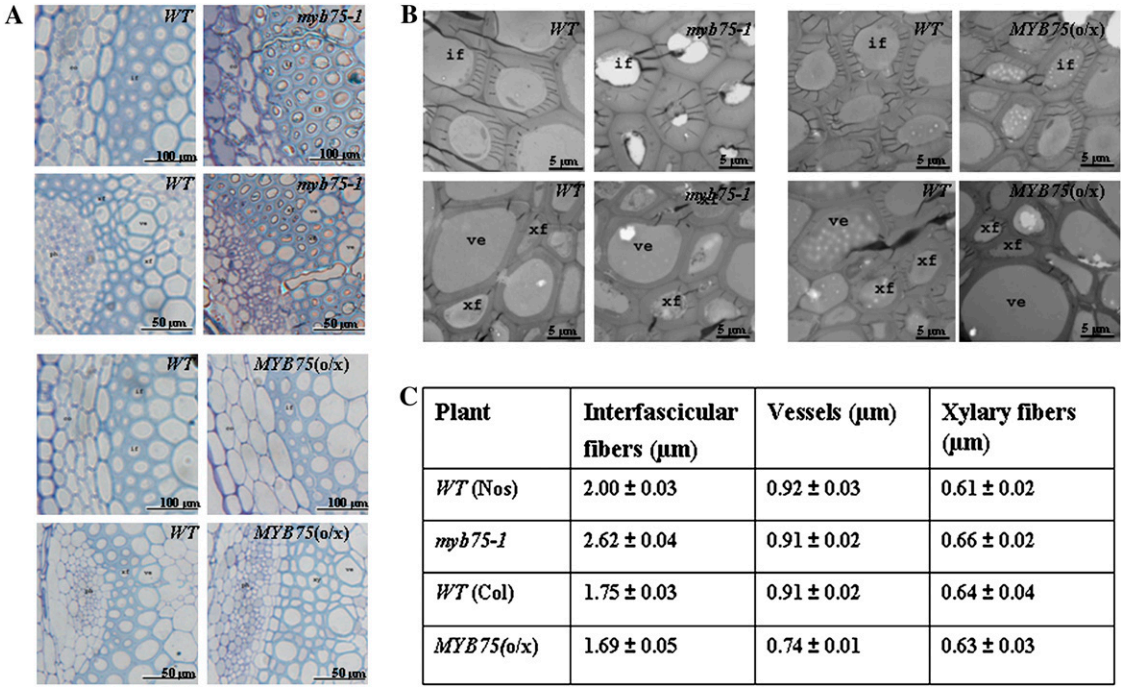


Figure 2. Nuclear localization and transcriptional activity of MYB75. A, Protoplast transfected with 35S:MYB75-YFP. Left, DIC image; middle, YFP channel; right, merged images. B, Effector and reporter constructs used in the transfection assays. C, Transcriptional activity of MYB75. Effector genes were made by fusing GD in-frame with MYB75. Effector gene plasmid DNA was cotransfected with reporter gene *Gal4*:*GUS* plasmid DNA into Arabidopsis leaf mesophyll protoplasts. GUS activity was assayed after the transfected protoplasts were incubated in darkness for 20 to 22 h. Shown are means ± SE of three replicates. MYB75 is a weak transcriptional activator.



C

Plant	Interfascicular fibers (μm)	Vessels (μm)	Xylary fibers (μm)
WT (Nos)	2.00 ± 0.03	0.92 ± 0.03	0.61 ± 0.02
myb75-1	2.62 ± 0.04	0.91 ± 0.02	0.66 ± 0.02
WT (Col)	1.75 ± 0.03	0.91 ± 0.02	0.64 ± 0.04
MYB75(o/x)	1.69 ± 0.05	0.74 ± 0.01	0.63 ± 0.03

Figure 3. Secondary wall thickening in fibers and vessels in *myb75-1* plants. The bottom inflorescence stem of 8-week-old plants were used for examination of secondary walls in fibers and vessels. A, Toluidine Blue staining of the cross sections in mutants and the wild type (WT; top; as indicated). Higher magnification of the Toluidine Blue-stained sections (bottom; as indicated). *myb75-1* show thickened cell wall compared to the wild type. B, TEM of the wild type, *myb75-1*, and *MYB75(o/x)*. *myb75-1* show thickened interfascicular fibers. C, Measurements of secondary cell wall thickness in the wild type and mutants (as indicated; μm). The wall thickness was measured from transmission electron micrographs of fibers and vessels. Data are means (μm) ± SE from 75 cells. if, Interfascicular fiber; ve, vessel; xf, xylary fiber. Bars = 50 and 100 μm (as indicated) in A and 5 μm in B.

from TEM micrographs confirmed that the interfascicular fiber wall thickness was increased in *myb75-1* plants, while little or no change was observed in vessel or xylary fiber wall thickness (Fig. 3C). No obvious differences were observed in the primary cell walls.

To determine if these changes in interfascicular fiber wall thickness might be associated with changes in cell wall chemistry, we assayed the Klason lignin content in mature inflorescence stems of both loss-of-function and gain-of-function mutant plants. Klason lignin content was significantly greater in stems of *myb75-1* plants but remained unaffected in the *MYB75(o/x)* genotype (Table I). Thioacidolysis was used to estimate the relative amounts of syringyl (S) and guaiacyl (G) monomers in the inflorescence stem lignin. This analysis revealed that the S/G monomer ratio was lower in *myb75-1* plants compared to the wild type, due primarily to lower levels of S subunits released by thioacidolysis (Table II). In the *MYB75(o/x)* genotype, the S/G ratio was higher than in the wild type, due both to an increase in released S units and a decrease in released G units (Table II). When we assayed changes in cell wall carbohydrate content in both *myb75-1* and *MYB75(o/x)* lines, no significant changes in Glc content were observed nor were significant differences detected in Gal, rhamnose, Man, or Ara content in either mutant (Table III).

Loss of *MYB75* Function Affects Expression of Genes Associated with Secondary Cell Wall Formation

Since manipulation of *MYB75* function led to changes in lignin content, we used qRT-PCR to examine the expression of genes encoding enzymes associated with phenylpropanoid metabolism and lignin monomer biosynthesis. This gene expression analysis focused on the lower inflorescence stems of the *myb75-1* and *MYB75(o/x)* lines and compared these with the respective wild-type backgrounds. Many of the genes examined were found to be up-regulated in the *myb75-1* stems (Fig. 4A), while their expression was generally unaffected in the *MYB75* overexpression background. We also assayed the expression of genes associated with cellulose and hemicellulose metabolism in secondary cell wall deposition or remodeling (Fig. 4B). While expression of primary cell wall-associated cellulose synthase genes (*CesA3*, *CesA5*, and *CesA6*) did not show any change in expression in either the loss-of-function or gain-of-function mutant, genes encoding the cellulose synthase isoforms (CesA4, 7, and 8) believed to be specifically responsible for biosynthesis of secondary cell wall cellulose microfibrils (Taylor et al., 2004) were strongly up-regulated in the *myb75-1* plant stems (Fig. 4B). In contrast to this impact on wall synthesizing systems,

Table I. *Lignin content in the lower stems of the wild type and mutants as determined by Klason analysis*

The analysis reveals a higher lignin content in *myb75-1* compared to the wild type. Each data point is the mean (mg/100 mg dry cell walls) ± SE of two separate assays.

Plant	Lignin
	mg/100 mg
Wild type (Nossen)	18.61 ± 1.01
myb75-1	23.24 ± 3.07
Wild type (Columbia)	21.09 ± 2.81
MYB75(o/x)	20.17 ± 2.89

the expression of *IFL1*, a gene regulating interfascicular fiber differentiation in Arabidopsis, or of *FRA8*, which encodes a putative glucuronyltransferase (essential for normal secondary wall synthesis) did not show any changes relative to wild-type stems. Interestingly, however, expression of two xylan biosynthetic genes, *IRX8* and *IRX9* (Pena et al., 2007; Persson et al., 2007), was increased in *myb75-1* (Fig. 4B). No reciprocal pattern of reduced gene expression of either phenylpropanoid or CesA genes was observed in the *MYB75* overexpression plants, relative to wild-type plants (Fig. 4).

MYB75 Physically Interacts with Other Transcription Factors Involved in Secondary Cell Wall Regulation

Several transcription factors whose expression in the Arabidopsis inflorescence stem is correlated with secondary cell wall deposition were identified in an earlier microarray study (Ehlting et al., 2005). Among these, MYB63 (Zhou et al., 2009) and KNAT7 (Brown et al., 2005; Zhong et al., 2008; Li, 2009) have since been demonstrated to be regulators of secondary wall formation. Since transcription factors are believed to often exert their regulatory activity through participation in multiprotein complexes, we asked whether any protein-protein interactions could be detected in directed yeast two-hybrid assays between MYB75 and the candidate regulators identified earlier by Ehlting et al. (2005). Within this assay matrix, positive yeast two-hybrid interactions were observed between MYB75 and KNAT7 and between TT8 and MYB63 (Fig. 5). MYB75 and TT8 have previously been shown to interact (Zimmermann et al., 2004), and this interaction therefore served as a positive control for these assays. All positive interactions detected were subsequently reconfirmed using different reporter genes (Supplemental Fig. S2), and the strength of the interactions was assayed using a chlorophenol red-β-D-galactopyranoside colorimetric reporter (Supplemental Fig. S3). The bimolecular florescence complementation (BiFC) assay using split YFP was used to demonstrate that the MYB75-KNAT7 interaction could also be observed in vivo in Arabidopsis protoplasts (Supplemental Fig. S4).

Photosynthetic Machinery and Cell Wall Modification Genes Are Up-Regulated in the *myb75-1* Loss-of-Function Mutant

To obtain a broader perspective on the possible role of MYB75 in carbon redistribution during inflorescence stem development in Arabidopsis, we compared the gene expression profiles of wild-type and *myb75-1* plants, with a specific focus on transcriptional activity in the maturing inflorescence stem. This analysis revealed that loss of MYB75 function resulted in both up-regulation and down-regulation of different gene sets in this tissue (Supplemental Table S2). Application of stringent cutoff values ($P < 0.05$ and fold change >2) excluded some secondary cell wall genes that had been earlier shown by qRT-PCR to be up-regulated in the *myb75-1* genotype (Fig. 4), but these lists included a number of up-regulated genes whose products are either predicted to be involved in cell wall modification or are important in other aspects of carbon metabolism, such as photosynthesis (Table IV). These include glycosyl hydrolases, a putative arabinogalactan-protein, several members of the light-harvesting protein complexes, LHCA1 (At3g54890), LHCB1 (At1g29910), LHCA3 (At1g61520), and LHCB3 (At5g54270), and genes encoding ribulose-bisphosphate carboxylase small subunit (At5g38420 and At1g67090). Differential expression of these genes was subsequently validated by qRT-PCR analysis (Supplemental Fig. S5).

DISCUSSION

Secondary cell wall deposition in plants is an important and dynamic phenomenon. Individual transcription factors involved in directly regulating secondary cell wall formation have been identified in previous studies (Zhong and Ye, 2007), but little is known about the common factors involved in secondary cell wall synthesis and in different carbon distribution pathways. MYB75 is a known regulator of anthocyanin accumulation in addition to its involvement in many other metabolic and environmental responses. It was shown here to act as a transcription factor that influences secondary cell wall formation in the maturing Arabidopsis inflorescence stems, where it impacts the lignin branch in particular. The regulation of cell wall deposition is particularly relevant for the inflorescence

Table II. *Lignin composition in the lower stems of the wild type and mutants as determined by thioacidolysis*

Analysis show changes in S/G ratio in *myb75-1* compared to the wild type. Each data point is the mean ± SE of four replicates.

Plant	Monomer Composition %		S/G
	G Lignin	S Lignin	
Wild type (Nossen)	55.61 ± 0.38	44.39 ± 0.40	0.78
myb75-1	58.94 ± 0.16	41.06 ± 0.18	0.67
Wild type (Columbia)	63.75 ± 0.18	36.25 ± 0.20	0.57
MYB75(o/x)	61.15 ± 0.56	38.85 ± 0.48	0.64

Table III. *Cell wall composition in the lower stems of the wild type and mutants determined by Klason analysis*

Each data point is the mean (mg/g dry cell walls) ± SE of two separate assays.

Carbohydrates	Wild Type (Nossen)	*myb75-1*	Wild Type (Columbia)	*MYB75(o/x)*
Glc	362.40 ± 1.77	371.80 ± 8.98	321.00 ± 14.84	291.70 ± 20.24
Ara	11.40 ± 3.56	12.20 ± 2.47	12.60 ± 1.65	10.80 ± 4.65
Xyl	104.00 ± 6.77	107.70 ± 2.29	97.10 ± 3.84	85.40 ± 8.67
Man	21.80 ± 5.50	20.20 ± 0.25	23.20 ± 5.63	21.70 ± 4.22
Rhamnose	9.40 ± 1.03	10.90 ± 3.53	13.4 ± 1.62	9.70 ± 1.50
Gal	18.20 ± 1.10	18.40 ± 1.53	18.30 ± 1.97	18.50 ± 2.91

stems in Arabidopsis because of the prominence in the mature stem of interfascicular fibers and xylem vessels that possess lignified secondary cells walls.

MYB75 was originally characterized as a transcriptional regulator promoting anthocyanin biosynthesis (Gonzalez et al., 2008) and much of that earlier work focused on phenotypes in seedlings and other juvenile tissues that display only limited commitment to secondary wall formation. In this study, we demonstrate a unique contribution of MYB75 to secondary cell wall biogenesis through its influence on lignin deposition, specifically in the inflorescence stem. It appears from our *MYB75* expression data that as the plant matures, the ubiquitous *MYB75* expression pattern reported previously in juvenile vegetative tissues becomes restricted to specific tissues. This observation is also consistent with the *MYB75* expression data from the AtGenExpress database (Schmid et al., 2005).

Cellulose synthesis in primary and secondary cell walls in Arabidopsis is believed to rely upon distinct members of the *CesA* gene family (Brown et al., 2005). A *MYB75* loss-of-function mutant displayed no obvious defects in primary cell wall formation but instead showed changes in the thickness of interfascicular fiber secondary cell walls and in cell wall chemistry of inflorescence stems in which secondary walls predominate. Consistent with a specific role for *MYB75* in the regulation of secondary cell wall biosynthesis, we observed *MYB75*-dependent regulation of a set of lignin biosynthetic genes (Fig. 4A) as well as those *CesA* genes thought to be dedicated to secondary cell wall synthesis (Fig. 4B). The role of MYB75 in inflorescence stem development appears to be restricted to secondary cell wall formation rather than more general regulation of tissue development within this organ, but we cannot exclude the possibility that MYB75 is involved in regulating other aspects of development not explored here.

Some secondary cell wall-associated MYB transcription factors, such as Arabidopsis MYB4 and MYB32,

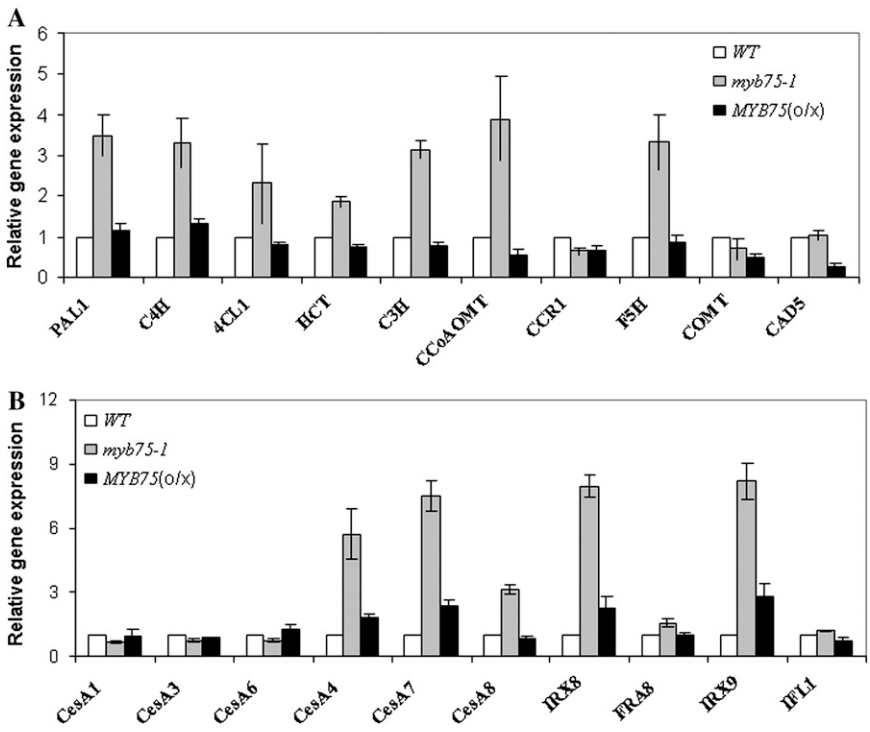


Figure 4. Secondary cell wall-associated gene expression in lower stems of *myb75-1* plants. A, qRT-PCR analysis of the expression of lignin biosynthetic genes in lower inflorescence stems of *MYB75* overexpresser [*MYB75(o/x)*] and loss-of-function mutants (*myb75-1*) compared with the wild type (WT). The expression levels of most of the genes in the lignin pathway were examined. 4CL1, 4-Coumarate-CoA ligase 1; HCT, hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase; C3H1, *p*-coumarate 3-hydroxylase 1. B, qRT-PCR analysis of the expression of secondary wall biosynthetic genes in lower inflorescence stems of *MYB75* overexpresser [*MYB75(o/x)*] and loss-of-function mutants (*myb75-1*) compared with the wild type. The expression levels of genes involved in the biosynthesis of cellulose in primary cell wall (*CesA1*, *CesA3*, and *CesA6*), cellulose in secondary cell wall (*CesA4*, *CesA7*, and *CesA8*), and xylan (*FRA8*, *IRX8*, and *IRX9*) were examined. Error bars represent SE of three replicates.

	AD-SND1	AD-AP2	AD-MYB63	AD-MYB20	AD-MYB5	AD-MYB75	AD-DST22
BD-KNAT7	-	-	-	-	+	+	-
BD-bZIP47	-	+	-	-	-	-	-
BD-TT8	-	-	+	-	+	+	-
BD-bHLH012	-	-	-	-	-	+	-
BD-DST32	-	-	-	-	-	-	-

Figure 5. In vitro protein-protein interactions among potential secondary cell wall-associated transcription factors and MYB75 as determined by yeast two-hybrid assay. Known interactions (shown in dark-gray box; Zimmermann et al., 2004) were used as a positive control, and empty vectors were cotransformed to be used as a negative control.

have been previously shown to act as negative transcriptional regulators of at least some steps in the phenylpropanoid pathway (Preston et al., 2004). Although loss of MYB75 function in the *myb75-1* mutant resulted in activation of genes encoding enzymes involved in lignin and cellulose biosynthesis, accompanied by an increase in interfascicular fiber cell wall thickness, our test of MYB75 trans-activation activity in a protoplast reporter system demonstrated that MYB75 displays only weak transcriptional activator activity and does not have repressor activity by itself within this assay system (data not shown). The weak transcriptional activation activity, or lack of transcriptional repression activity, of MYB75 in the trans-action reporter assays employed in our study could be due to the absence from mesophyll protoplasts of appropriate interaction partners that affect MYB75 function. In addition, KNAT7 repression observed in this system could be due to steric hindrance. Alternatively, MYB75 could be required for the activation of unknown downstream regulators that themselves function to repress expression of genes encoding secondary cell wall biosynthetic genes. The effect of altered *MYB75* transcript levels on the relative amounts of syringyl and guaiacyl monomer subunits released by thioacidolysis of inflorescence stem lignin is consistent with earlier evidence for the contribution of caffeic acid *O*-methyltransferase (COMT) and CCoAOMT activities to phenolic ring methylation (Do et al., 2007). In *myb75-1*, CCoAOMT is up-regulated, while COMT is unchanged, a pattern that could have resulted in synthesis of more G subunits and less S-type lignin (Table II).

In contrast to our findings, Borevitz et al. (2000) reported that *MYB75* overexpression in whole Arabidopsis plants resulted in increased expression of core phenylpropanoid genes with no impact on the S/G ratio of the lignin. This discrepancy in lignin monomer ratios might be related to the differences in lignin analysis methodology (thioacidolysis versus derivatization) used in the two studies. More importantly, the two studies examined very different types of tissue, with the Borevitz et al. (2000) analyses being carried out on whole plants, while our study focused on the mature inflorescence stem. Consistent with a profound developmental effect on the function of MYB75, we observed that gene expression changes induced by *MYB75* overexpression in *MYB75(o/x)* seedlings differed markedly from the patterns observed in inflorescence stems and more closely resembled those reported for whole plants by Borevitz et al. (2000) and Tohge et al. (2005b; Supplemental Fig. S6). It is clear, therefore, that the regulatory influence of transcription factors in plant tissues can be spatially and temporally conditioned.

The biosynthesis of secondary cell wall components is thought to be a highly integrated and coordinated process in which changes in the biosynthesis or regulation of an individual component can compromise the overall assembly or composition of the wall. For example, there is experimental evidence that a reduction in any of the three major secondary wall components, cellulose, xylan, or lignin, can result in a reduction in secondary wall thickening (Zhong et al., 1998, 2005; Taylor et al., 2004; Pena et al., 2007). The increased secondary wall thickening phenotype observed in the *myb75-1* mutants could therefore be an indirect effect associated with increased lignin deposition.

The data presented here suggest that one role of MYB75, in addition to regulating aspects of phenyl-

Table IV. *Expression changes of genes related to the photosynthetic machinery and cell wall modification in the inflorescence stem of myb75-1 plants*

Locus	Gene Name	Gene Description	Pathway	Fold Change
At3g54890	LHCA1	Light-harvesting chlorophyll *a*/*b*-binding protein	Carbon metabolism	5.30
At1g29910	LHCB1.2	PSII type I chlorophyll *a*/*b*-binding protein	Carbon metabolism	4.04
At1g61520	LHCA3	Light-harvesting chlorophyll *a*/*b*-binding protein	Carbon metabolism	3.37
At5g54270	LHCB3	Light-harvesting chlorophyll *a*/*b*-binding protein	Carbon metabolism	3.06
At5g38420	RBCS-2B	Ribulose-bisphosphate carboxylase small subunit 2b	Carbon metabolism	2.88
At1g67090	RBCS1A	Ribulose-bisphosphate carboxylase small unit-related	Carbon metabolism	2.50
At1g45130	BGAL5	Glycosyl hydrolase family 35 (β-galactosidase)	Cell wall modification	2.52
At5g10430	AGP4	Arabinogalactan-protein	Cell wall modification	2.27
At5g26000	BGLU38	Glycosyl hydrolase family 1, myrosinase precursor	Cell wall modification	2.20

propanoid metabolism, is in more generally regulating secondary cell wall formation in the Arabidopsis stem. Loss of *MYB75* function results in the channeling of carbon toward the lignin pathway, generating increased lignin accumulation in secondary cell walls, whereas constitutive overexpression of *MYB75* leads to activation of anthocyanin biosynthesis-related genes and enhanced carbon flow into the flavonoid pathway. It remains possible, however, that the visible increase in anthocyanin production (Borevitz et al., 2000) associated with overexpression of MYB75 does not reflect its endogenous function, since overexpression of transcription factors can potentially generate artefactual pleiotropic phenotypes. Nevertheless, several MYBs have been shown earlier to be involved in activation of anthocyanin biosynthesis (Gonzalez et al., 2008), and MYB75 may be contributing to this overall regulatory activity. In addition, transcript profiles (Table IV) from the *myb75-1* inflorescence stems reflect carbon flux redistribution within the branches of phenylpropanoid metabolism as well as into other metabolic pathways.

Many positive yeast two-hybrid interactions of different strengths were observed among the potential transcription factors (Fig. 5; Supplemental Figs. S2 and S3) tested from different transcription factor families. In particular, the protein-protein interaction of MYB75 with other transcription factors involved in secondary cell wall regulation is consistent with a model in which MYB75 acts as a member of one or more transcriptional regulatory complexes. Whether it might serve as an activator or repressor of transcriptional targets within such putative complexes in planta remains to be clarified. It is noteworthy that the interaction of MYB75 with TT8, a bHLH protein (Zimmermann et al., 2004), was previously shown to involve a multiprotein complex that regulates anthocyanin production (Gonzalez et al., 2008). Our observation that MYB75 also interacts with KNAT7 (Fig. 5; Supplemental Figs. S2 and S4), a transcription factor shown to play a role in secondary cell wall formation in Arabidopsis (E. Li, S. Wang, J.-G. Chen, and C.J. Douglas, unpublished data; Brown et al., 2005; Li, 2009) and also nuclear localized (Li, 2009), suggests a scenario in which multiple complexes might share specific sets of transcription factors, thus providing a rich palette of combinatorial diversity for cross-regulating different metabolic pathways as both activators and repressors of transcription.

MATERIALS AND METHODS

Plant Material

The Arabidopsis (*Arabidopsis thaliana*) loss-of-function allele of MYB75 (*myb75-1*; pst16228) is the result of a *Ds* insertion at the *MYB75* locus, in the Nossen ecotype background (Kuromori et al., 2004) and was obtained from the RIKEN Bioresource Centre (http://rarge.gsc.riken.jp/dsmutant/index.pl This allele has been genetically characterized earlier and demonstrated to possess a *Ds* insertion tightly linked to the MYB75 phenotype (Teng et al., 2005). We further confirmed this insertion in the *MYB75* locus and identified plants homozygous for this insertion by PCR screening. Primer sequences for amplification of the flanking fragments and genotyping of the insertion lines were as follows: left primer (LP), 5′-TGGTTTTGTAGGGCTAAACCG-3′, and right primer (RP), 5′-AAACACCGGATACATACCTTTTTC-3′. To amplify the flanking fragment, LP was combined with Ds5-3 (5′-TACCTCGGGTTC-GAAATCGAT-3′), and RP was combined with Ds3-2a (5′-CCGGATCG-TATCGGTTTTCG-3′). For genotyping the insertion, primers LP, RP, and Ds 3-2a were used. The wild-type line produces a PCR product of approximately 900 bp (from LP to RP) on a 1% agarose gel. Lines carrying the homozygous insertion produce an approximate 500-bp band (from RP to Ds 3-2a), while heterozygous lines produce both bands. The PCR amplification program was as follows: (1) one cycle of 94°C for 2 min; (2) 30 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 3 min; and (3) 72°C for 10 min. The gain-of-function mutant for *MYB75* [*MYB75(o/x)*; Borevitz et al., 2000] is an activation-tagged mutant (*pap-1D*) in the Columbia background and was obtained from the Arabidopsis Biological Resource Center. Homozygous plants of each genotype were used for all experiments, along with appropriate wild-type plants (*myb75-1* versus Nossen and *pap1-D* versus Columbia) as comparison controls. Seeds were surface sterilized using 20% commercial bleach, cold treated at 4°C in the dark for 2 d, and plated on half-strength MS agar medium (2.16 g/L MS salts, 1% Suc, and 1% Bacto-agar pH 6.0 adjusted with 1 M KOH; Murashige and Skoog, 1962). Ten-day-old seedlings were grown in 5 × 5-cm pots containing a moistened Sunshine Mix #1 (Sun Gro Horticulture Canada),with a 16/8-h (light/dark) photoperiod at approximately 120 μmol m^{-2} s^{-1} and a temperature of 23°C, unless specified otherwise. Growth comparisons were performed on 6-week-old plants grown at the above-described conditions. For TEM, microscopy, and qRT-PCR experiments, inflorescence stems were harvested from 8-week-old plants, and the lower half of the stem was used for analysis. For chemical analyses, whole stems were dried in a 50°C oven overnight and ground in a Wiley mill to pass a 40-mesh screen.

Analysis of Anthocyanin Content

Seedling anthocyanin content was determined using a procedure modified from that of Neff and Chory (1998). In short, at least two groups (50 seedlings each) of 8-d-old seedlings from each genotype were extracted overnight in 150 μL of methanol acidified with 1% HCl. After the addition of 100 μL of distilled water and 250 μL of chloroform, anthocyanins were separated from chlorophylls by solvent partitioning. Total anthocyanin content in the aqueous phase was determined spectroscopically by measuring the A_{530} and A_{657}. By subtracting the A_{657} from the A_{530}, the relative amount of anthocyanin per seedling was calculated [$(530 - A_{657}) \times 50$ seedling^{-1}].

GUS Reporter Gene Analyses

Transgenic Arabidopsis plants (T2) generated earlier by Gonzalez et al. (2008) were employed in this study. They express a *MYB75pro*::*GUS* construct derived from the 2.2-kb genomic DNA region upstream of the MYB75 coding sequence in the Columbia ecotype, fused with the GUS coding sequence. Histochemical analysis of the GUS reporter gene expression was performed as described previously (Malamy and Benfey, 1997) using different plant organs, as well as transverse hand sections of inflorescence stems of 6- to 8-week-old T2 *MYB75pro*::*GUS* transgenic plants.

Protoplast Isolation, Transfection, and GUS Activity Assay

Leaves from Columbia wild-type plants approximately 3 to 4 weeks old were used for protoplast isolation and subsequent transfection and GUS activity assays, as described previously (Wang et al., 2007). For GUS activity assays, the plasmid DNAs for reporter and effector genes were isolated using Endofree Plasmid Maxi Kits (Qiagen). Additional GD plasmid DNA was used to equalize the amount of DNA in each plasmid preparation. A 10-μg aliquot of each effector plasmid and 10 μg of reporter plasmid were used in co-transfection assays. Each transfection assay was performed in triplicate, and each experiment was repeated at least twice

Localization of MYB75-YFP

To examine the localization of MYB75-YFP in protoplasts, the Gateway recombination cassette (Invitrogen) from pEARLYGATE104 (Earley et al., 2006) was used as a destination vector for generation of an N-terminal YFP fusion of MYB75. The resulting plasmid was transfected into freshly prepared

Arabidopsis leaf mesophyll protoplasts and incubated for 20 to 22 h (Wang et al., 2007). YFP fluorescence was visualized using a Leica DM-6000B upright fluorescence microscope with phase and differential interference contrast (DIC) and photographed with a Leica FW4000 digital image acquisition and processing system (Leica Microsystems).

BiFC Using YFP

For generation of N-terminal YFP-tagged constructs, the appropriate entry clone was transferred into BiFC expression vector pCL112 (pBATL) to produce nYFP-vectors. The same procedure was used for C-terminal YFP-tagged constructs using pCL113 (pBATL) to produce cYFP vectors. The resulting plasmids were cotransfected into freshly prepared Arabidopsis leaf mesophyll protoplasts and incubated for 20 to 22 h (Wang et al., 2007). YFP fluorescence was examined and photographed using a Leica DM-6000B upright fluorescence microscope with phase and DIC equipped with a Leica FW4000 digital image acquisition and processing system (Leica Microsystems).

qRT-PCR

For organ-specific expression analyses, total RNA was isolated from different organs of 6-week-old Arabidopsis (Nossen wild type) plants (three biological replicates, each consisting of pooled tissues from 8–10 plants), and qPCR was performed as described below. Relative values are arbitrary units and were calculated as described previously (Gutierrez et al., 2008). For the secondary cell wall-specific and lignin-specific gene expression study, total RNA for real-time PCR was isolated from the lower half of inflorescence stems [wild type, *myb75-1*, and *MYB75(o/x)*] with three biological replicates (each consisting of pooled stems from 8–10 plants) using the RNeasy Mini Protocol (Qiagen). To eliminate residual genomic DNA, the RNA was treated with RNAse-free DNAseI according to the manufacturer's instructions (Qiagen). The concentration of RNA was quantified using the A_{260}, and the quality of the sample preparation was assessed using the A_{260}/A_{280} ratio. Total RNA (2 μg) was reverse transcribed using the SuperScript VILO cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. cDNA was diluted (1:20), and 2 μL was used in each reaction in a 20-μL reaction volume. PCR amplification was performed with gene-specific primers for secondary cell wall or lignin-specific genes (Supplemental Table S1) using *Actin8* as a normalization control (Supplemental Table S1). The cDNA was amplified using the PerfeCta qPCR FastMix (Quanta Biosciences) on the DNA Engine Opticon 2 (Bio-Rad). Differences in gene expression, expressed as fold change relative to control, were calculated using the $[\Delta][\Delta]C_t = 2^{[\Delta]Ct,Actin-[\Delta]Ct,gene}$ method. Each measurement was carried out in triplicate, and the error bars represent SE of the mean of fold changes for the three biological replicates.

Bright-Field and TEM

Tissue for light microscopy and TEM was harvested from the inflorescence stem (5 cm from the base) of 8- to 10-week-old plants and fixed with glutaraldehyde. Fixed stems were vacuum infiltrated in 1% osmium tetroxide and 0.05 M sodium cacodylate (pH 6.9) for 30 min, rinsed twice, and then dehydrated through an aqueous alcohol series (30%–100%; 15 min for each dilution). Dehydrated stems were soaked twice in anhydrous acetone before embedding in low viscosity Spurr's resin. Sections (0.5 μm) were cut using a Leica Ultracut T and Druuker diamond Histoknife and stained with Toluidine Blue for bright-field microscopy (using an Olympus AX70 microscope) or with uranyl acetate for TEM (viewed on a Hitachi H7600 PC-TEM).

For cell wall thickness measurements, the width of the secondary cell wall in micrographs obtained from TEM was quantified in 75 cells for each genotype using ImageJ software (http://rsbweb.nih.gov/ij/).

Chemical Analysis

Lignin content was determined by a modified Klason method, according to Coleman et al. (2008), in which solvent-extracted ground Arabidopsis stem tissue (0.1 g) was treated with 3 mL 72% H_2SO_4 for 2 h at room temperature and then diluted to 3% H_2SO_4 and autoclaved for 60 min. The concentrations of different monosaccharides in the acid hydrolysate were determined using HPLC (DX-500; Dionex) equipped with an anion-exchange PA1 (Dionex) column, a pulsed amperometric detector with a gold electrode, and a SpectraAS3500 autoinjector (Spectra-Physics). Each analysis was run in duplicate. The monosaccharides were separated on the PA1 column with water at a flow rate of 1 mL/min, and the eluate received a postcolumn addition of 200 mM NaOH (0.5 mL/min) prior to detection.

Thioacidolysis was performed as described by Robinson and Mansfield (2009), and the reaction products were analyzed by gas chromatography.

Microarray Analysis

Total RNA was extracted from Arabidopsis inflorescence stems (Nossen wild type and *myb75-1*) using a Qiagen Plant Mini RNA extraction kit. The quantity and quality of total RNA were assessed on the Agilent 2100 Bioanalyzer (Agilent Technologies) using the Agilent RNA 6000 Nano kit and reagents. Samples of total RNA (10 μg) for six wild-type and six *myb75-1* biological replicates were reverse transcribed using a SuperScript II RT kit (Invitrogen) and the appropriate 3DNA primers (cyanine5- or cyanine3-specific capture sequences) to achieve dye balance with two technical replicates for each of three biological replicate pairings. The 3DNA Array 350 kit (Genisphere) was used according to manufacturer specifications for cDNA hybridizations, and subsequent 3DNA (dendromer) fluorescent probe hybridizations onto custom-made full-genome (30 K) Arabidopsis 70-mer oligo arrays (Douglas and Ehlting, 2005; Ehlting et al., 2005) printed at the Prostate Centre Microarray Facility, Vancouver. Hybridizations were carried out using a Slidebooster SB401 (Advalytix) according to Array 350 specifications, and the hybridized slides were scanned with a ScanArray Express (Perkin-Elmer). Scanned images were quantified using Imagene software (BioDiscovery), and the resulting data were analyzed in the R package using Bioconductor tools and custom scripts. For background correction, the mean of the dimmest 5% of spots in a particular subgrid (grouping of 26 × 27 spots) was used as the background value for the spots in that subgrid. Background-corrected spot intensities were then normalized on each array using the robust local-linear regression algorithm LOWESS (or LOESS) included in the R package, with a span of 0.7 (Yang et al., 2002). The relative expression ratio for each gene represents the average of three biological replicates, where P value significance estimates were computed using a two-tailed Student's t tests ($\alpha = 0.05$) and adjusted for false discovery rate using a q-value correction based upon Storey (2002).

Yeast Two-Hybrid Assays

The ProQuest yeast two-hybrid system (Invitrogen) was used with full-length transcription factors in pDEST32 (bait vector) or pDEST-22 (prey vector) and introduced into the yeast strain MaV203 in different combinations. Positive clones were isolated on the basis of three selectable markers: *HIS3*, *URA3*, and *LacZ*. Positive interactions were indicated by activation of *HIS3* or *URA3*, according to the manufacturer's instructions. To compare the strength of the protein-protein interactions, quantitative assays for β-galactosidase activity in liquid cultures were performed using chlorophenol red-β-D-galactopyranoside as a substrate according to the manufacturer's instructions.

GenBank database accession numbers for the genes investigated in this study are MYB75 (At1g56650), MYB63 (At1g79180), MYB20 (At1g66230), PAL1 (At2g37040), C4H (At2g30490), 4CL1 (At1g51680), HCT (At5g48930), C3H1 (At2g40890), CCoAOMT1 (At4g34050), CCR1 (At1g15950), F5H1 (At4g36220), COMT (At5g54160), CAD5 (At4g34230), CesA4 (At5g44030), CesA7 (At5g17420), CesA8 (At4g18780), IRX8 (At5g54690), IRX9 (At2g37090), CesA1 (At4g32410), CesA3 (At5g05170), CesA6 (At5g64740), FRA8 (At2g28110), and IFL1 (At5g60690).

Supplemental Data

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

Supplemental Figure S1. Activation-tagged and Ds insertion mutants of *MYB75*, anthocyanin content and growth, and inflorescence stem phenotype.

Supplemental Figure S2. Confirmation of yeast two-hybrid interactions by different reporter genes.

Supplemental Figure S3. Quantification of strength of yeast two-hybrid interactions by the CPRG assay.

Supplemental Figure S4. Confirmation of yeast two-hybrid interactions by BiFC assay in Arabidopsis protoplasts

Supplemental Figure S5. qRT-PCR validation of expression of candidate genes identified by microarray analysis as up-regulated (>2-fold; P =

0.05; Table I) in the lower inflorescence stem of *myb75*, relative to Nossen-wild type.

Supplemental Figure S6. Real-time quantitative PCR analysis of the expression of lignin biosynthetic genes in *MYB75* overexpresser [*MYB75(o/x)*] seedlings compared with the wild type.

Supplemental Table S1. PCR primers used in the study.

Supplemental Table S2. Genes up-regulated or down-regulated in the wild type versus *myb75-1* microarray study ($P < 0.05$ and fold change >2).

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