The MYB305 Transcription Factor Regulates Expression of Nectarin Genes in the Ornamental Tobacco Floral Nectary

Guangyu Liu, Gang Ren,1 Adel Guirgis,2 and Robert W. Thornburg3
Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, Iowa 50011

We have isolated and characterized the cDNA encoding the ornamental tobacco (Nicotiana langsdorffii X N. sanderae) homolog of the antirrhinum (Antirrhinum majus) MYB305. This transcription factor was robustly expressed at Stage 12 of nectary development but was only weakly expressed in the earlier Stage 6 nectaries. The ornamental tobacco MYB305 contains a conserved R2R3 MYB DNA binding domain with 76 amino acids in the activation domain. A green fluorescent protein-MYB305 fusion localized to nucleus of tobacco protoplasts and yeast one-hybrid assays demonstrated that it functions as a transcription activator. A conserved 23–amino acid C-terminal domain is required to activate gene expression. The coding region of the myb305 cDNA was expressed in Escherichia coli as a glutathione S-transferase fusion protein and was purified to homogeneity. This protein shows binding to two consensus MYB binding sites on the ornamental tobacco Nectarin I (nec1) promoter as well as to the single site located on the Nectarin V (nec5) promoter. Deletions of either of the binding sites from the nec1 promoter significantly reduced expression in nectary tissues. Temporally, MYB305 expression precedes that of nec1 and nec5, as would be expected if the MYB305 factor regulates expression of the nec1 and nec5 genes. Ectopic expression of MYB305 in foliage was able to induce expression of both nec1 and nec5, as well as two flavonoid biosynthetic genes in the foliage. Finally, RNA interference knockdown of MYB305 resulted in reduced expression of both nectarins and flavonoid biosynthetic genes. We conclude that expression of MYB305 regulates expression of the major nectarin genes in the floral nectary.

INTRODUCTION

Many species of angiosperms attract animals (insects, birds, and mammals) to visit their reproductive organs and physically transfer pollen from anthers, where it is produced, to the stigma where it begins the fertilization process. On the surface, reliance on other organisms to mediate sexual fertilization seems an unsatisfactory strategy; however, the wide diversity and great success of the angiosperms that have evolved over the past 125 million years punctuates the tremendous success of this strategy. Plants attract these visiting pollinators to the flower by offering a reward of nutritionally rich nectar, which is produced from an unusual floral organ, the nectary, that typically is located at the base of the flower near the ovary. Floral nectar accumulates in the cup-like environment formed at the floral base (Nepi, 2007). Although nectar is an aqueous solution of sugars, it also contains amino acids (Carter et al., 2006) as well as a variety of other components. Krebs cycle intermediates, lipids, vitamins, proteins, and biologically important metal cations have all been identified in nectar from a variety of species (Nicolson and Thornburg, 2007).

Because visiting pollinators are promiscuous and are not sterile, the plant’s reproductive tract, which is bathed in this metabolically rich nectar, is at high risk for microbial infection. Teleologically, few places in the plant are as important as the ovary, where the next generation’s seeds will develop. Therefore, plants must have mechanisms to defend the ovary from microbial attack. We have identified a number of proteins (termed nectarins) that accumulate in the soluble nectar of ornamental tobacco (Nicotiana langsdorffii X N. sanderae) plants (Carter et al., 1999; Carter and Thornburg, 2000, 2004b, 2004c; Naqvi et al., 2005). Understanding the biochemistry of these proteins has led to the identification of the nectar redox cycle, a complete biochemical pathway expressed in the soluble nectar of ornamental tobacco plants that produces a defense substance. We propose that this pathway has evolved to defend the gynoecium from invading microorganisms (Thornburg et al., 2003; Carter and Thornburg, 2004a; Carter et al., 2007).

The nectar redox cycle involves the production of very high levels of hydrogen peroxide: up to 4 mM (Carter and Thornburg, 2000). This pathway is initiated by a developmentally regulated, nectary-expressed NADPH oxidase that begins producing superoxide just prior to anthesis (Carter et al., 2007). The superoxide is subsequently disproportionated into oxygen and hydrogen peroxide by the major nectar protein, Nectarin I (NEC1; Carter and Thornburg, 2000). Among the other nectar proteins, Nectarin V (NEC5) is a glucose oxidase that also produces hydrogen peroxide but via a different mechanism from NEC1 (Carter and Thornburg, 2004c). Because of the highly oxidative nature of

1 Current Address: Department of Plant Pathology, Pennsylvania State University, 0419 Life Sciences Building, University Park, PA 16802 USA
2 Current Address: Institute of Genetic Engineering and Biotechnology, Menofiya University, PO Box 79/22857, Sadat City, Egypt
3 Address correspondence to thorn@iastate.edu.

Some figures in this article are displayed in color online but in black and white in the print edition.

Online version contains Web-only data.

www.plantcell.org/cgi/doi/10.1105/tpc.108.060079
ornamental tobacco nectar, antioxidants (both ascorbate and β-carotene) are important in nectary function (Carter and Thornburg, 2004b; Homer et al., 2007). Other nectarins function to limit fungal invasion of the gynoecium by inhibiting fungal enzymes that degrade plant cell walls (Naqvi et al., 2005).

While nectar proteins have been long reported (Beutler, 1935; Zimmerman, 1954), their study at the molecular level is still in its infancy. The floral nectarins of only two species, ornamental tobacco (Nicotiana rustica) and ornamental tobacco nectar, antioxidants (both ascorbate and β-carotene) are important in nectary function (Carter and Thornburg, 2004b; Homer et al., 2007). Other nectarins function to limit fungal invasion of the gynoecium by inhibiting fungal enzymes that degrade plant cell walls (Naqvi et al., 2005).

For NEC1, the major nectar protein, we previously evaluated the transcriptional programs regulate their expression (Carter et al., 1999; Carter and Thornburg, 2004b, 2004c; Naqvi et al., 2005). This consensus MYB binding site within the nec1 promoter in transgenic plants (Carter and Thornburg, 2003). From deletion studies, we identified a consensus MYB binding site within the nec1 promoter and predicted that a MYB transcription factor was involved in the temporal expression of NEC1 and possibly NEC5 as well (Carter and Thornburg, 2003). This consensus MYB binding site was previously identified in flavonoid biosynthetic genes, including phenylalanine ammonia lyase (pal) and chalcone isomerase (chi) of antirrhinum (Antirrhinum majus), bean (Phaseolus vulgaris), and parsley (Petroselinum crispum; Sablowski et al., 1994). Two MYB transcription factors, MYB305 and MYB340, were shown to bind to this sequence and to regulate expression of flavonoid biosynthetic genes in antirrhinum flowers. It is interesting to note that the A. majus MYB305 gene was also highly expressed in nectaries (Moyano et al., 1996).

In other gene expression studies, we performed an EST study of gene expression at three different stages of ornamental tobacco nectary development (D.-L. Yin, K. Taylor, and R.W. Thornburg, unpublished results). One outcome of this study was the identification of a highly expressed MYB transcription factor in the nectary. This study was therefore designed to investigate a potential role of this highly expressed MYB transcription factor in regulating NEC1 and NEC5 expression.

RESULTS

The myb305 cDNA from LxS8 ornamental tobacco plants was cloned as part of a nectary EST project. Because we had previously suggested that a MYB transcription factor was responsible for the temporal activation of the nec1 promoter in tobacco nectaries (Carter and Thornburg, 2003), we characterized this factor in detail.

Expression of MYB305

To determine where this myb gene was expressed, we initially examined various whole-plant tissues by RT-PCR to evaluate gene expression. As seen in Figure 1, this myb gene is strongly expressed only in flowers. It was not expressed in stems (lane 6), roots (lane 8), or leaves (lane 12). In reproductive tissues, this myb gene was expressed in the ovary (lane 5), the floral tube (lane 7), and petals (lane 13) but was most strongly expressed in the floral nectary (lanes 1 to 4). This myb gene was not detected in anthers (lane 9), stamens (lane 10), or sepals (lane 11). Based on this analysis, we conclude that in ornamental tobacco, this myb gene was expressed uniquely in flowers with the highest level in the nectary and with lower levels in the ovary, floral tube, and petals.

Phylogenetic Analysis

BLAST analysis (Altschul et al., 1990) of the translated protein sequence clearly identifies it as an R2R3 MYB transcription factor (e value = 2e-74) and reveals that this protein is most closely related to a group of MYB proteins that includes the A. majus MYB transcription factors MYB305 and MYB340 (Jackson et al., 1991), the Pisum sativum MYB26 (Ulimar and Strommer, 1997), and the Gerbera hybrida MYB8 (Laitinen et al., 2005). Because Am-MYB305 was the first of this group of proteins to be identified (Jackson et al., 1991), we named this protein the N. langsdorffii X N. sanderae (LxS) MYB305.

To place this gene into a phylogenetic context, we analyzed the MYB DNA binding domains of a group of MYB proteins, including a representative subset of 37 of the 132 Arabidopsis thaliana R2R3 MYB proteins and six Antirrhinum MYB proteins using the neighbor joining method. The 37-protein Arabidopsis subset was chosen to cover all phylogenetic space but to simplify the analysis by limiting the number of individual proteins in this analysis (Kranz et al., 1998; Stracke et al., 2001). As seen in Figure 2A, the MYB DNA binding domains identify a distinct clade of proteins containing LxS-MYB305 and other closely related proteins, including one each from G. hybrida and P. sativa and two each from A. majus (Am-MYB305 and Am-MYB340) and

Figure 1. Temporal and Spatial Expression of myb305 in LxS8 Plants.

RNAs were isolated from individual tissues and processed for RT-PCR as described in Methods. Lane 1, stage 2 nectary; lane 2, stage 6 nectary; lane 3, stage 9 nectary; lane 4, stage 12 nectary; lane 5, ovary at anthesis; lane 6, stem; lane 7, floral tube at anthesis; lane 8, root; lane 9, anthers at anthesis; lane 10, stamens at anthesis; lane 11, sepals at anthesis; lane 12, leaf; lane 13, petals at anthesis. Cycle number for both myb305 and gapdh is 24 for all tissues tested.

Figure 2A. Phylogenetic Analysis of MYB Proteins from Myb305 and Related Proteins.

BLAST analysis of the translated protein sequence clearly identifies it as an R2R3 MYB transcription factor (e value = 2e-74) and reveals that this protein is most closely related to a group of MYB proteins that includes the A. majus MYB transcription factors MYB305 and MYB340 (Jackson et al., 1991), the Pisum sativum MYB26 (Ulimar and Strommer, 1997), and the Gerbera hybrida MYB8 (Laitinen et al., 2005). Because Am-MYB305 was the first of this group of proteins to be identified (Jackson et al., 1991), we named this protein the N. langsdorffii X N. sanderae (LxS) MYB305.

To place this gene into a phylogenetic context, we analyzed the MYB DNA binding domains of a group of MYB proteins, including a representative subset of 37 of the 132 Arabidopsis thaliana R2R3 MYB proteins and six Antirrhinum MYB proteins using the neighbor joining method. The 37-protein Arabidopsis subset was chosen to cover all phylogenetic space but to simplify the analysis by limiting the number of individual proteins in this analysis (Kranz et al., 1998; Stracke et al., 2001). As seen in Figure 2A, the MYB DNA binding domains identify a distinct clade of proteins containing LxS-MYB305 and other closely related proteins, including one each from G. hybrida and P. sativa and two each from A. majus (Am-MYB305 and Am-MYB340) and

Figure 2A. Phylogenetic Analysis of MYB Proteins from Myb305 and Related Proteins.

BLAST analysis of the translated protein sequence clearly identifies it as an R2R3 MYB transcription factor (e value = 2e-74) and reveals that this protein is most closely related to a group of MYB proteins that includes the A. majus MYB transcription factors MYB305 and MYB340 (Jackson et al., 1991), the Pisum sativum MYB26 (Ulimar and Strommer, 1997), and the Gerbera hybrida MYB8 (Laitinen et al., 2005). Because Am-MYB305 was the first of this group of proteins to be identified (Jackson et al., 1991), we named this protein the N. langsdorffii X N. sanderae (LxS) MYB305.

To place this gene into a phylogenetic context, we analyzed the MYB DNA binding domains of a group of MYB proteins, including a representative subset of 37 of the 132 Arabidopsis thaliana R2R3 MYB proteins and six Antirrhinum MYB proteins using the neighbor joining method. The 37-protein Arabidopsis subset was chosen to cover all phylogenetic space but to simplify the analysis by limiting the number of individual proteins in this analysis (Kranz et al., 1998; Stracke et al., 2001). As seen in Figure 2A, the MYB DNA binding domains identify a distinct clade of proteins containing LxS-MYB305 and other closely related proteins, including one each from G. hybrida and P. sativa and two each from A. majus (Am-MYB305 and Am-MYB340) and
Arabidopsis proteins (At-MYB21 and At-MYB24). In addition, At-MYB57 appears near the root of this clade. These three Arabidopsis genes are collectively known as the Family 19 clade of MYB genes (Kranz et al., 1998; Stracke et al., 2001). Based upon MPSS analysis (mpss.udel.edu/at/), both At-MYB21 and At-MYB24 are exclusively expressed in the MPSS inflorescence libraries, while At-MYB57 is expressed in flowers and in a variety of other tissues.

To get an enhanced picture of this clade, the full protein sequences (DNA binding domain plus activation domain) of the eight most closely related genes were phylogenetically analyzed and are displayed in Figure 2B. As can be seen, LxS-MYB305 more closely resembles G. hybrida MYB8 than the A. majus MYBs or P. sativa MYB26. The association of the Arabidopsis proteins with this group suggests that the MYB305 family proteins may share some functions with At-MYB Family 19 proteins; however, the Arabidopsis proteins also appear to form a separate subclade within this group, and this clade separation suggests that the MYB305 family genes may have unique functions.

**Nuclear Localization of MYB305**

To evaluate the subcellular localization of MYB305, we used a transient transformation assay using tobacco mesophyll protoplasts. For these studies, the green fluorescent protein (GFP) reporter gene was fused in frame to the 5' end of the LxS-MYB305 open reading frame (Figure 3A). The plasmid pMDC43 encoding GFP alone served as a control. Protoplasts transformed with pMDC43 showed GFP fluorescence throughout the entire cell (Figure 3B). By contrast, cells transformed with the full-length GFP::MYB305 construct showed bright fluorescence localized to the nucleus (Figure 3C). To investigate which part of the MYB protein possessed the nuclear localization signal, LxS-MYB305 was truncated into two parts, MYB305Δ1 and
MYB305 is a Transcription Activator in Yeast

To investigate whether MYB305 can serve as a transcriptional activator, we used yeast one-hybrid assays to drive the expression of marker genes (Shioda et al., 1997). Initially, the full-length LxS-MYB305 coding sequence was fused to the 3' end of the GAL4 DNA binding domain to generate the effector construct pRT624 (Figure 4A). The reporter construct contains a modified his3 gene in which a gal1' upstream activation sequence has been inserted into the his3 promoter. Positive and negative controls were a reconstructed GAL4 (GAL4DB-AD) and a construct containing only the GAL4 DNA binding domain (pDBleu). All constructs were transformed into the yeast strain MaV203 (Vidal et al., 1996) that harbors his3' reporter systems (Figure 4A) as well as a similar lacZ reporter. All constructs were assayed visually using dilution growth tests, and expression was quantitated using the lacZ reporter system.

As shown in Figure 4B, little growth was observed when the pDBbleu strain (negative control) was grown on medium lacking histidine. By contrast, strong growth was observed with the reconstructed GAL4 strain (positive control) at 10^{-3} and even 10^{-4} dilutions. The effector construct, pRT624, that encoded the GAL4 DNA binding domain fused with the complete MYB305 protein also resulted in strong growth of transgenic yeast. It also gave ~20-fold more β-galactosidase activity than the negative control in the quantitative lacZ reporter assay (Figure 4C). Based upon these data, we conclude that the LxS-MYB305 is capable of activating transcription in yeast.

MYB305 Activates Transcription via Its Conserved C Terminus

Because MYB305 is a transcription activator, we sought to identify those portions of the molecule responsible for transcription activation. Therefore, we prepared a series of DNA constructs that lacked various portions of the activation domain. These constructs, also shown in Figure 4A, contain the entire R2R3 domain but no activation domain (amino acids 1 to 125; pRT631), the complete activation domain but no R2R3 domain (amino acids 108 to 192; pRT632), the C-terminal half of the activation domain (amino acids 158 to 192; pRT633), and the entire MYB305 protein lacking only the C-terminal 30 amino acids (amino acids 1 to 162; pRT635). All constructs were transformed into the yeast strain MaV203 and were assayed by dilution growth tests and quantitated with the lacZ reporter system.

As shown in Figure 4B, yeast cells harboring pRT631, which lacks the complete activation domain, do not grow well on media lacking histidine. By contrast, each of the constructs that contains the C-terminal end of the activation domain (pRT632, pRT633, and pRT634) all grow quite well on media lacking histidine, indicating that sequences in the C-terminal region of the MYB305 are responsible for transcriptional activation. To confirm this, we tested pRT635 containing the complete protein sequence except for the final 30 amino acids. This construct did not grow well on the media lacking histidine, confirming that the C terminus of the protein mediates transcriptional activation. Quantitation of the β-galactosidase activity in these yeast strains (Figure 4C) demonstrated that pRT632, pRT633, and pRT634 provide 10- to 20-fold greater transcriptional activity than the control strain, pDBleu. Activity measured from constructs lacking either the entire activation domain (pRT631) or the final 30
Amino acids (pRT635) of that domain were not above background level.

Acidic Residues Near the MYB305 C Terminus Mediate Transcriptional Activation

To evaluate further the function of the activation domain, we analyzed its predicted secondary structure (amino acids 117 to 192) using Jpred, a neural network-based program (Cuff and Barton, 1999, 2000) that identifies structural homologs, performs multiple sequence alignment, and classifies each residue as likely to reside in an α-helix, β-sheet, or random coil secondary structure. As shown in Figure 5A, the algorithm identified and aligned the same transcription factors found in our earlier BLAST analyses. This alignment also shows that the C-terminal region required for transcriptional activation identified in construct pRT634 (black bar above the sequences) is the most conserved feature of these transcription factors. The Jpred secondary structure prediction algorithm identified only one feature shared among the aligned activation domains, a single α-helix that is seven amino acids long, residing near the C terminus. The remaining portions of the activation domains of these transcription factors were predicted to be unstructured.

The predicted α-helix lies near the center of the sequence that is required for transcriptional activation. When we mapped amino acid residues from this region of LxS-MYB305 onto a helical structure (Figure 5B), the hydrophobic residues Met-180, Ile-183, Trp-184, and Met-186 lie on one face of the α-helix, while the hydrophilic residues Glu-181, Asp-182, and Ser-185 are located on the opposite face of the helix, suggesting this may be a classical amphipathic α-helix. Because amphipathic α-helices often present important amino acid side chains for biological activity, we hypothesize that the conserved amphipathic nature of this helix contributes to the transcriptional activation function in these proteins.

Short acidic sequences are well known to activate transcription (Ruden et al., 1991). To evaluate the potential role of acidic sequences in the activation activity of the LxS-MYB305 C-terminal domain, we made a series of site-directed mutations designed to alter acidic amino acids. In addition to the Asp and Glu residues that occur in this region, acidic charges can also be generated by phosphorylation of amino acids in this portion of the molecule. Phosphorylation is a common posttranslational modification of transcription factors (Magasanik, 1988; Decker and Kovarik, 1999) and the online analysis tool NetPhos (Blom et al., 1999) predicted that Ser-185 in the C-terminal domain of LxS-MYB305 may be phosphorylated. Therefore, we prepared a series of modified C-terminal domains fused to the GAL4 DNA binding domain. In construct pRT648, both Ser-179 and Ser-185 were changed to Ala. In construct pRT653, the acidic residues Glu-181, Asp-182, and Asp-192 were changed to Ala. In

**Figure 4.** LxS-MYB305 Is a Transcriptional Activator in Yeast and It Activates the Transcription of Reporter Genes via Its C-Terminal Domain.

(A) Constructs used in these studies. The GAL4 DNA binding domain (DB) and LxS-MYB305-∆ fragment (MYB-∆) fusion genes driven by the ADH1 promoter serve as effectors; the HIS3 and LacZ (data not shown) genes with Gal4 DNA binding site on their promoters serve as reporters. The constructs of each effector are shown with DNA fragments identified above the individual constructs. R2 and R3 represent the R2 and R3 repeat of MYB DNA binding domains, respectively; AD represents the activation domain. The + control contains a recombined Gal4 DNA binding domain and activation domain; the – control contains Gal4 DNA binding domain only.

(B) Serial dilution growth assays of transformed yeast on selective YSM His– + 10 mM 3AT medium. Constructs are identified to the left of the figure. Dilutions are identified below the figure.

(C) Quantitation of β-galactosidase activity in yeast. Constructs are identified below the bars (means ± SD, n = 4 with two replicates for each sample). †, positive control construct; ‡, negative control construct. [See online article for color version of this figure.]
pRT654, all five residues were changed to Ala. As shown in Figure 5C, changing the Ser residues to Ala in construct pRT648 had no effect on the transcriptional activation of the modified protein, suggesting that phosphorylation of these Ser residues may not be important for transcriptional activation. By contrast, changing the acidic residues to Ala in either construct pRT653 or pRT654 completely abolished the transcriptional activation activity of the fusion protein. We conclude that acidic residues located near the MYB305 C terminus mediate transcriptional activity of this transcription factor.

**MYB305 Binds the nec1 and nec5 Promoters in Vitro**

NEC1 is the major protein secreted into nectar of ornamental tobacco (Carter et al., 1999; Carter and Thornburg, 2000). Cloning of the nec1 promoter and subsequent promoter deletion analysis revealed the presence of a consensus MYB DNA binding site located at \(-899\) to \(-891\) of the promoter (Carter and Thornburg, 2003). Deletion of this site resulted in loss of reporter gene expression in the nectary. Based upon these analyses, we previously predicted the involvement of a MYB protein in nec1 transcriptional activation. In this study, we reexamined the nec1 promoter sequence and identified a second potential MYB binding site located at \(-86\) to \(-78\) of the nec1 promoter (Figure 6B). To evaluate whether LxS-MYB305 can bind to these regions of the nec1 promoter, we expressed this transcription factor as a glutathione S-transferase (GST) protein fusion in *Escherichia coli* and purified it by virtue of the GST tag. The GST-MYB305 fusion protein was purified to a single protein band as visualized on SDS-PAGE (Figure 6A, lane 2). We also developed antisera against the LxS-MYB305 for use in these studies. This antiserum reacts with the GST-MYB305 fusion protein (lane 4) but not against GST alone (lane 5). The antibodies also recognize the native MYB305 in nectary tissues (lane 6) but show no reactivity against any proteins present in the foliage of the ornamental tobacco plants (lane 7).

This purified GST-MYB305 fusion protein was then used in electrophoretic mobility shift assays to evaluate its interactions with \([^{32}P]\)-labeled DNA probes. Two duplex oligonucleotide probes were used for these analyses, the S1 probe (N1S1; see Supplemental Table 1 online) corresponds to the upstream MYB binding site \((-899\) to \(-891\)) and the S0 probe (N1S0) corresponds to a second binding site \((-86\) to \(-78\)) within the nec1 promoter. The GST protein alone cannot bind either S1 or S0.
probes (Figures 6C and 6D), whereas GST-MYB305 can bind both labeled probes. In each case, the addition of GST-MYB305 resulted in a substantial mobility shift of the probe. The addition of 10-fold excess unlabeled S1 or S0 probe successfully competed for the binding of GST-MYB305 to the respective labeled probe. We also tested whether the addition of a 10-fold excess of an unlabeled, modified competitor C1 (N1C1) or C0 (N1C0) probe that lacked the MYB binding site could displace the interaction of GST-MYB305 with the original labeled probes. In each case, the competitor probes, either C1 or C0, were identical to the S1 and S0 probes with the exception that the nine-nucleotide MYB consensus sequence was replaced with a polyA/T track.

Figure 6. MYB305 Binds to the nec1 and nec5 Promoter Fragments.

(A) Expression and purification of LxS-MYB305 protein. Lanes 1 to 3 are Coomassie blue stained: lane 1, protein standards; lane 2, purified GST-MYB305 protein; lane 3, GST protein. Lanes 4 to 7 are immunoblots using purified anti-LxS-MYB305 antibodies: lane 4, purified GST-MYB305 protein; lane 5, GST protein; lane 6, 100 μg of protein from Stage 12 nectary tissue of wild-type LxS8 plant; lane 7, 100 μg of protein from foliage of wild-type LxS8 plant.

(B) Structure of the N. plumbaginifolia nec1 promoter showing the location and sequences of the two consensus MYB binding sites S1 and S0.

(C) Mobility shift of the radiolabeled S1 probe. Inclusion of individual components is indicated below each lane. S1 probe represents the S1 MYB binding site on the nec1 promoter. The unlabeled S1 probe is identical to the radiolabeled S1 probe and the unlabeled C1 probe differs from S1 probe with MYB consensus sequence replaced by poly A/T as indicated in Supplemental Table 1 online. Film exposure was 2 h.

(D) Mobility shift of the radiolabeled S0 probe. Inclusion of individual components is indicated below each lane. S0 probe represents the S0 MYB binding site of the nec1 promoter. The unlabeled S0 probe is identical to the radiolabeled S0 probe, and the unlabeled C0 probe differs from S1 probe with MYB consensus sequence replaced by poly A/T as indicated in Supplemental Table 1 online. Film exposure was 2 h.

(E) Structure of the N. plumbaginifolia nec5 promoter showing the location of the consensus MYB binding site.

(F) Mobility shift of the fluorescently labeled N5P probe. Inclusion of individual components is indicated below each lane. The unlabeled N5P probe is identical to the fluorescently labeled N5P probe, and the unlabeled N5C probe differs from N5P probe as indicated in Supplemental Table 1 online. [See online article for color version of this figure.]
(see Supplemental Table 1 online). As shown in Figure 6, the addition of these unlabelled competitor probes had no effect on the binding of the GST-MYB305 to the labeled S1 and S0 probes. Based on these observations, we conclude that LxS-MYB305 binds specifically to the S1 and S0 MYB binding sites in vitro.

The regulation of the Nectarin V (nec5) promoter is similar to that of the nec1 promoter, and there is a single consensus MYB binding site located at -448 of the nec5 promoter (Figure 6E; Carter and Thornburg, 2003). Therefore, we tested the purified GST-MYB305 for interaction with a synthetic nec5 promoter element. As shown in Figure 6F, GST-MYB305 did indeed interact specifically with the nec5 MYB binding site. This binding could be competed with an excess of unlabelled N5P probe but not with the unlabelled mutant probe (N5C). These observations led us to conclude that in addition to binding the nec1 promoter, LxS-MYB305 also interacts with the MYB binding site in the nec5 promoter.

The patterns of mobility shifts observed in Figures 6C and 6D show multiple interactions of the DNA probes with GST-MYB305, suggesting a possible multimerization of the proteins interacting with the probes. GST is known to dimerize in vitro (Lim et al., 1994), and the observed patterns may be due to GST multimerization. To test whether MYB305 could form a homodimer, we used the yeast two-hybrid method. We fused the first 162 amino acids of LxS-MYB305 lacking the final 30 amino acids of the activation domain with the yeast two-hybrid bait vector, pDEST32 (www.invitrogen.com), and the full-length LxS-MYB305 to the two-hybrid prey vector, pDEST22, respectively. No activity was detected in the two-hybrid interaction (see Supplemental Figure 1 online), and we conclude that the truncated 162–amino acid LxS-MYB305 protein does not homodimerize in the yeast two-hybrid assay and that multimerization is likely due to the presence of the GST in the GST-MYB305 fusion protein. However, because the bait construct lacked the C-terminal 30 amino acids, we cannot unequivocally conclude that the intact protein does not dimerize through this small C-terminal region.

**Wild-Type, but Not Mutant, nec1 Promoters Drive β-Glucuronidase Expression**

To evaluate the role of the MYB binding sites in regulating transcription from the nec1 promoter, we generated a series of promoter-β-glucuronidase (GUS) constructs that contained the wild-type nec1 promoter or the nec1 promoter with a mutation at the S1 site, at the S0 site, or at both sites (Figure 7, inset). These promoter-GUS constructs were mobilized into Agrobacterium tumefaciens LBA4404 and used to transform N. tabacum cv Xanthi plants. After selection, the plants were grown to floral maturity. Subsequently, nectaries were isolated and tested for nec1 promoter function. As shown in Figure 7, four transgenic plants, each harboring the wild-type nec1 promoter-GUS fusion construct, all expressed between 2300 and 4400 units of GUS activity per mg nectary protein. By contrast, when either the S1 site, the S0 site, or both sites were mutated, there was a dramatic reduction in GUS activity expressed in the nectaries of each of four independent transformants prepared from each construct, suggesting that both the S1 and S0 consensus MYB binding sites are both important for promoter activation. These data are consistent with our earlier observation that deletion of the upstream site results in a dramatic loss of promoter activity (Carter and Thornburg, 2003).

**Temporal Expression of myb305, nec1, and nec5**

We have provided evidence that MYB305 may function as a transcriptional regulator of nec1 expression. The LxS-MYB305 protein is nuclear localized, it activates transcription in yeast, it exists in a dramatic loss of promoter activity (Carter and Thornburg, 2003).
Tobacco flower development previously has been divided into 12 morphologically distinct stages between the mature bud stage (stage S1) and anthesis (S12) (Koltunow et al., 1990). To evaluate when myb305 is expressed, we identified stage-specific flowers, isolated the nectaries, extracted stage-specific mRNA, and performed RT-PCR for myb305 as well as for nec1 and nec5 mRNAs. We used 18S and 26S RNA as loading and PCR controls.

As shown in Figure 8A, myb305 is expressed by floral stage S6 and remains on until floral stage S12 (anthesis). These results are similar to the results shown in Figure 1. After fertilization, myb305 is abruptly downregulated. By contrast, both nec1 and nec5 begin to be expressed after stage S8 (~36 h later). Although nec1 is more strongly expressed than nec5, they both continue to be expressed, accumulating to high levels at anthesis (stage S12). These results are entirely consistent with our earlier observations on the expression patterns of nec1 and nec5 (Carter et al., 1999; Carter and Thornburg, 2003, 2004c). Figure 8B shows MYB305 and NEC1 protein levels by immunoblot. These protein blots confirm the expression of myb305 and nec1 in Figure 8A. Together, these results demonstrate that myb305 is expressed prior to nec1 and nec5 and that MYB305 protein accumulates in the nectary prior to expression of both the nec1 transcript and the NEC1 protein. Thus, we conclude that expression of myb305 temporally precedes the expression of nec1 and nec5 in developing nectaries.

**Figure 8. Temporal Expression of myb305 in Nectaries of Wild-Type LxS8 Plants.**

(A) RT-PCR of myb305, nec1, and nec5 in the nectary glands of LxS8 plants at different floral stages. Both 18S and 26S rRNAs were used as controls. PF = 48 h postfertilization. Amplification cycle number for 18S rRNA, 26S rRNA, and myb305 is 24 for all floral stages. Amplification cycle number for nec1 and nec5 is 26 for all floral stages.

(B) Protein blotting of MYB305 and NEC1 proteins in the nectary glands of LxS8 plants at different floral stages. Film exposure for MYB305 was 20 min. Film exposure for NEC1 was 30 min.

**Ectopic Expression of MYB305 Causes Foliar Expression of nec1 and nec5**

To test further the functionality of MYB305 in nectarin gene expression, we expressed the LxS-myb305 cDNA in the foliage of *N. tabaccum* plants and tested whether this ectopic expression was able to drive expression of nectarin genes in leaves. In addition, two genes in the flavonoid biosynthetic pathway, pal and chi, have previously been shown to be regulated by the *A. majus* MYB305 (Sabolowski et al., 1994, 1995), so we also evaluated expression of these genes in the foliage of these plants.

For this study, we developed four independent transgenic overexpressor (OX) plant lines that express the coding region of LxS-myb305 under the control of the strong cauliflower mosaic virus 35S promoter. The phenotype of the OX plants was similar to the wild-type plants, with the exception that they were shorter, growing to ~50% the height of wild-type plants (see Supplemental Figure 2 online). As shown in Figure 9A, quantitative RT-PCR analysis demonstrates that none of the genes of interest were expressed in the foliage of wild-type, untransformed tobacco plants. These results confirm the expression pattern of myb305 shown in Figure 1 as well as the published patterns of expression previously observed for nec1 and nec5 (Carter et al., 1999; Carter and Thornburg, 2003, 2004c). As opposed to the wild-type plants, the myb305 mRNA is detectable in the foliage of each of the overexpression lines. Similarly, nec1 and the nec5 transcripts were found in the overexpression lines. One line, OX1, showed a very weak signal for nec5, while all other lines showed substantial levels of nec1 and nec5 expression in the MYB305 overexpression lines. In addition, we also tested the expression of the flavonoid genes pal and chi. Neither of these flavonoid genes was expressed in the foliage of wild-type tobacco plants (Figure 9A), but both were observed in each of the MYB305 overexpression lines. Based on these results, we conclude that ectopic expression of each of the target genes (nec1, nec5, pal, and chi) in the foliage correlates with the expression of myb305. In contrast with nec1 and nec5, neither Nectarin III (nec3) nor Nectarin IV (nec4) were expressed in the foliage of these plants (see Supplemental Figure 3 online), suggesting that these genes are not regulated by MYB305. These results for MYB305 and NEC1 were confirmed at the protein level by immunoblot analysis. Wild-type plants showed undetectable levels of both MYB305 and NEC1 (Figure 9C). Again, these results are internally consistent with our earlier observations (Figure 7A) and with previously published observations (Carter et al., 1999, 2007). Similarly, MYB305 and NEC1 proteins were readily observed in the OX plants, especially OX3 and OX4. The OX1 and OX2 plants showed low levels of MYB305 and NEC1 and required long exposures to observe the bands. The wild-type plants failed to show any bands, even with these long exposures.

**RNA Interference Knockdowns of MYB305 Also Knocks Down Expression of nec1 and nec5**

Finally, we also prepared a series of RNA interference (RNAi) lines that showed reduced expression of myb305 in the nectary. Quantitative RT-PCR analysis of Stage 12 nectaries from wild-type...
plants (Figure 9B) shows strong expression of myb305, both nec1 and nec5, as well as both of the flavonoid biosynthetic genes (pal and chi) in the wild-type plants. Again, these data are consistent internally with our earlier observations (Figures 1 and 8) as well as with previously published observations (Moyano et al., 1996; Carter et al., 1999; Carter and Thornburg, 2003, 2004c). In each of the four independent RNAi knockdown lines, nectary expression of myb305 is reduced. Similarly, both nec1 and nec5 are also reduced in the nectaries of these knockdown lines, as are both of the flavonoid biosynthetic genes. Thus, we conclude that a reduction in the level of myb305 expression correlates with reduced levels of nec1 and nec5 expression as well as of pal and chi expression. As before, we also examined the expression of MYB305 and NEC1 at the protein level using immunoblots (Figure 9D). While the wild-type plants showed substantial amounts of both proteins, each of the RNAi knockdown lines showed reduced levels of the proteins in Stage 12 nectaries, confirming that the protein levels accumulating in the

---

**Figure 9.** Analysis of LxS-MYB305 Overexpression and RNAi Plants.  
(A) Quantitative RT-PCR analysis of LxS-myb305, nec1, nec5, pal, and chi expression in the foliage of LxS-MYB305 overexpression N. tabacum plants. Means + SD are shown (four biological replicates with two technical replicates each).  
(B) Quantitative RT-PCR analysis of LxS-myb305, nec1, nec5, pal, and chi expression in the stage 12 nectary glands of LxS-MYB305 RNAi N. tabacum plants. Means + SD are shown (four biological replicates with two technical replicates each).  
(C) Immunoblotting of MYB305 and NEC1 proteins in the foliage of LxS-MYB305 overexpression N. tabacum plants. Film exposure time was 1 h for MYB305 and 12 h for NEC1.  
(D) Immunoblotting of MYB305 and NEC1 proteins in the stage 12 nectary glands of LxS-MYB305 RNAi N. tabacum plants. Film exposure for both MYB305 and NEC1 was 30 min.  
(E) Phenotype comparison of wild-type N. tabacum nectary and RNAi N. tabacum nectary. Bar = 2 mm.
nectaries of these RNAi plants reflect the levels of nectary-expressed myb305 and nec1 mRNAs.

**Additional Phenotypes Associated with the MYB305 RNAi and OX Plants**

In addition to the molecular phenotypes observed in the RNAi lines and OX lines (i.e., myb305, nec1, nec5, pal, and chi expression), we also have observed additional phenotypes that affect the nectary, flower, and the whole plant. The phenotypes of RNAi lines include failure of the nectary to accumulate high levels of β-carotene (Figure 9E), a considerably reduced flow of nectar, failure of the flower petals to expand at anthesis, decreased pigmentation in the petals, an elongated style, and an abscission phenotype in which the entire floral bud falls from the stalk. Because these phenotypes are unrelated to the role of MYB305 in regulating the nectarin genes, these additional phenotypes will be the subject of a future, more detailed investigation of these plants. The ectopic expression of MYB305 results in a unique plant shape phenotype: the OX plants grow slower, are dwarf, branch earlier, and have crinkly leaves (see Supplemental Figure 2 online). The broad nature of these additional phenotypes suggests that MYB305 has roles in floral development that go beyond flavonoid metabolism and nectar protein expression.

**DISCUSSION**

In earlier work, we identified a consensus MYB binding site within the nec1 promoter and hypothesized that a MYB family transcription factor might activate the temporal expression of the nec1 gene in vivo (Carter and Thornburg, 2003). In this study, we identified MYB305 as a major nectary-expressed transcription factor. We have shown that this factor is expressed exclusively in flowers and have presented evidence that this protein is the transcription factor that directly binds to and activates transcription of the both the nec1 and nec5 promoters. Phylogenetically, LxS-MYB305 is closely related to snapdragon MYB305 and homologs from G. hybrid and P. sativa, as well as to a group of MYB proteins from Arabidopsis (MYB21, MYB24, and MYB57).

This group of proteins has long been thought to function in the activation of flavonoid biosynthetic genes. Am-MYB305 was first identified in 1991 in Antirrhinum flowers (Jackson et al., 1991). It was later shown to regulate transcription of the pal, chi, and 4-coumaryl-CoA-ligase genes in snapdragon flowers (Sablowski et al., 1994). Another related MYB protein, MYB340, is functionally redundant in snapdragon flowers (Moyano et al., 1996) and is closely related to this group of proteins (Figure 2). These two proteins, MYB305 and MYB340, appear to be regulated by phosphorylation in Antirrhinum flowers (Moyano et al., 1996). When the Am-MYB305 protein was expressed in foliage, ectopic expression of flavonoids was observed (Sablowski et al., 1995). In pea, MYB26 binds the P-box-like binding sites in the promoter regions of several flavonoid biosynthetic genes (Uimari and Strommer, 1997). Gh-MYB8 interacts with bHLH factor GMYC1 and is required for activation of a late anthocyanin biosynthetic gene promoter PGDFR2 (Laitinen et al., 2005). So activation of genes in the flavonoid and anthocyanin pigmentation pathways may be a general feature of these proteins.

The Arabidopsis MYB21 and MYB24 genes are involved in jasmonate response during stamen development (Mandaokar et al., 2006). In Arabidopsis, they are required for proper anther development (Li et al., 2006; Mandaokar et al., 2006). It has also been shown that overexpression of MYB24 causes aberrant anther development (Yang et al., 2007). MYB21 is a flower-specific gene, but in cop1 mutants, MYB21 is expressed throughout the seedling tissues (Shin et al., 2002), although MYB24 is not (Li et al., 2006). Whether cop1 regulates the myb305 genes in other species is not clear and because cop1 mutants are not available in snapdragon, gerbera, tobacco, or pea, this may remain unclear for some time.

Because the Arabidopsis genes appear to function in anther development, we also carefully examined anther length in the flowers of our wild-type LxS-MYB305 RNAi plants as well as the LxS-MYB305 OX plants. While the anthers all appeared to be morphologically normal, they were affected in their length (see Supplemental Table 2 online). Both the OX and the RNAi lines had shorter anthers than the wild type, with the RNAi lines being more severely affected. Likewise, the style length for each of the mutant lines was also shorter than the wild type; however, style length showed a more severe reduction with the OX lines. Thus, while both anther and style lengths were affected by both overexpression of MYB305 as well as by knockdown of MYB305 expression, there was not a clear direct association of MYB305 expression with the phenotype. Whether the related MYB305 proteins in other species also function in anther development is unknown. None of these earlier papers dealing with MYB305 that have been published over the past 15 years has mentioned the role of these proteins in anther development; however, this does not rule out the possibility that these proteins could have marginal function in anther development as observed in tobacco.

We have also examined nectaries of the Arabidopsis myb21 and myb24 mutant lines as well as the myb21 and myb24 double mutant lines (Mandaokar et al., 2006), but we have not observed any clear nectary phenotypes in these mutants. Thus, although they share high identity, the relationship of the myb305 genes with the Arabidopsis Family 19 MYB genes is far from clear. However, it is interesting to note that the MYB26 protein sequence from P. sativa (a rosid) clusters together with the sequence from ornamental tobacco (an asterid) rather than the sequences from Arabidopsis (another rosid). To confirm the robustness of this phylogeny, we analyzed the ornamental tobacco sequence together with only the rosid sequences (from pea and Arabidopsis). As shown in Supplemental Figure 4 online, the pea sequence still clusters with the ornamental tobacco sequence rather than with the other rosid sequences. This further highlights the differences between the myb305 family genes and the Arabidopsis Family 19 MYB genes and suggests that the myb305 genes may have additional functions. It is noteworthy that the myb305 family genes are identified in species that produce relatively large quantities of nectar as opposed to Arabidopsis, which produces very little nectar (Davis et al., 1998). Whether the additional functions of MYB305 relate to high levels of nectar production is currently under investigation.
We demonstrated that the LxS-MYB305 protein accumulates in nuclei of transiently transformed tobacco protoplasts and that the nuclear localization signal is localized in the R2R3 domain. *Arabidopsis* MYB24 has also been shown to be nuclear (Yang et al., 2007). Within the nucleus, these proteins interact with the promoters of several flavonoid and nectarin genes. We demonstrated that the LxS-MYB305 protein functions as a transcriptional activator in yeast one-hybrid assays, confirming its potential role as a transcriptional regulator. A 75-amino acid C-terminal domain, when fused with the GAL4 DNA binding domain, strongly activated transcription. A series of deletions within this C-terminal domain more precisely localized the transcriptional activation domain to a 23–amino acid region at the C terminus of the protein.

Alignments of the related MYB proteins indicated that this 23–amino acid region at the C terminus is the most conserved portion of the activation domain. This conservation among MYB proteins has been previously reported (Kranz et al., 1998; Li et al., 2006). However, this study evaluates the structural features of this region and reveals new insights. Secondary structure predictions suggested that the activation domain lacks defined secondary structure, with the single exception of a predicted short α-helix located near the middle of the 23–amino acid activation region. This α-helix was found to be amphipathic with hydrophilic residues exposed on one side of the helix and hydrophobic residues on the other. Such α-helices are often strong activation regions in transcription factors (Bushman et al., 1989).

This 23–amino acid region was found to contain a number of acidic residues, as well as two Ser residues that could be phosphorylated to provide additional acidic residues in this region. Short acidic peptide sequences are well known to activate transcription when brought into proximity of DNA (Ma and Ptashne, 1987). In the case of LxS-MYB305, we found that a short 23–amino acid peptide was all that was required to activate transcription when fused to the GAL4 DNA binding domain. This identified peptide has a net excess of four acidic residues. Site-specific mutagenesis revealed that Ala substitutions of two Ser residues did not alter transcriptional activation; however, replacement of three acidic residues with Ala residues completely abolished transcription.

To determine whether LxS-MYB305 could bind specific motifs within the nec1 promoter, we expressed the protein as a GST fusion and tested the purified protein for direct interaction with the MYB binding sites in the nec1 promoter. For both of the S1 and S0 MYB binding sites in the nec1 promoter, unlabeled probe competed for binding of the GST-MYB305 protein to the labeled duplex oligonucleotide probes, but unlabeled mutated probes did not compete, indicating that the interaction was specific for the probe sequence. Similarly, GST-MYB305 also bound the MYB binding site in the nec5 promoter. Unlabeled duplex probes also competed for this binding, but unlabeled mutated probes did not.

To evaluate further the role of these MYB binding sites, we prepared a series of GUS reporter constructs that contained either the wild-type or mutagenized nec1 promoters. In transgenic plants, we found that the wild-type nec1 promoter expressed GUS activity at appreciable levels in four independent transformants; however, deletion of either or both of the MYB binding sites from the nec1 promoter resulted in loss of promoter activity. The finding that both sites are required to achieve high levels of expression is somewhat surprising and suggests that multiple MYB-DNA interactions may be required to achieve high-level activation of the nec1 promoter. Further studies along these lines will be needed to reveal the role of these interactions in transcriptional activation of these promoters.

In addition, we have generated transgenic plant lines that show ectopic expression of LxS-MYB305. These plants also showed ectopic expression of the flavonoid genes, *pal* and *chi*, in foliage as expected (Sablowski et al., 1994; Moyano et al., 1996; Uimarri and Strommer, 1997; Laitinen et al., 2005) as well as ectopic expression of the nectarin genes. Both nec1 and nec5 were coordinately expressed in the foliage of these plants; however, nec3 and nec4 were not (see Supplemental Figure 3A online). Based upon differential patterns of expression, we had previously predicted that nec3 and nec4 were expressed via different mechanisms from nec1 and nec5 (Carter and Thornburg, 2004b; Naqvi et al., 2005), and these studies confirm this.

Finally, we generated RNAi lines that show severely reduced levels of LxS-MYB305. These lines each show reduced accumulation of nec1 and nec5 transcripts in nectaries. These lines also show reduced expression of the flavonoid genes *pal* and *chi*. In addition, the nectaries of these RNAi plants do not accumulate high levels of β-carotene as wild-type nectaries do. These plants also show reduced levels of nectar accumulation in the flower. Both of these characteristics (β-carotene accumulation and nectar production) are associated with starch metabolism in nectaries (Horner et al., 2007; Ren et al., 2007a, 2007b). The role of LxS-MYB305 in nectary starch metabolism is currently an active project in our laboratory.

In this manuscript, we provide evidence that MYB305 is a transcriptional regulator that functions in expression of Nectarin I and Nectarin V. LxS-MYB305 is expressed most strongly in the nectary, but it also is expressed in the ovary, floral tube, and petals. The protein is nuclear localized, it activates transcription in yeast, and it binds to the nec1 and nec5 promoters in vitro. Furthermore, mutation of the MYB binding sites within the nec1 promoter destroys promoter activity in vivo, and myb305 is transcribed prior to expression of the target genes nec1 and nec5. In addition, ectopic expression of LxS-MYB305 in foliage results in foliar expression the target genes nec1, nec5, *pal*, and *chi* and reduced expression of LxS-myb305 via RNAi results in a coordinated knockdown of these same target genes nec1, nec5, *pal*, and *chi*. Based on all of these data, we conclude that in addition to regulating the flavonoid biosynthetic genes, MYB305 also regulates the nec1 and nec5 genes in the nectaries of ornamental tobacco. Furthermore, the additional phenotypes observed in the RNAi knockdown and overexpression plants suggest that LxS-MYB305 has roles in floral development that are far beyond flavonoid metabolism and nectar protein expression.

**METHODS**

**Materials**

Unless otherwise noted, the materials used in these studies were obtained from either Fisher Chemical or Sigma-Aldrich Chemical and were of the highest quality available. All radioisotopes were from Perkin-Elmer.
Plants

The LxS8 ornamental tobacco (Nicotiana tabacum) plants used in these studies, growth condition of plants, and methods for isolation of nectar and floral tissues were previously described (Carter et al., 1999). If not used immediately, tissues were frozen at −20°C until use. Flowers were staged as described (Kull et al., 1990). N. tabacum cv Xanthi plants were also previously described (Thornburg et al., 1987). The Arabidopsis thaliana myb21, myb24 mutants, and the double mutant (Mandaokar et al., 2006) were kindly supplied by John Browse, Washington State University.

Genes

Complete sequencing was performed on both strands of all clones at the Iowa State University Nucleic Acid Facility. Synthesis of the various cDNA libraries used to produce the LxS-myb305 cDNAs was previously described (Carter and Thornburg, 2004c; Naqvi et al., 2005).

Anti-LxS-MYB305 and Anti-NEC1 Antiserum

Rabbit anti-NEC1 antiserum was previously described (Carter et al., 1999). Rabbit anti-LxS-MYB305 antiserum was produced by GenScript using a synthesized polypeptide, H2N-PFLTETNDNIWSMED-COOH, 1999). Rabbit anti-Rabbit anti-NEC1 antiserum was previously described (Carter et al., 1999). Rabbit anti-LxS-MYB305 antiserum was produced by GenScript using a synthesized polypeptide, H2N-PFLTETNDNIWSMED-COOH, which corresponds to the C terminus of the LxS-MYB305 protein. The antiserum was affinity purified prior to use. The peptide was coupled to CNBr-activated Sepharose 4 resin (GE Healthcare). Twenty milliliters of crude antiserum were passed through the column and after washes with 100 ml 50 mM Tris-HCl, pH 7.0, 100 ml 10 mM Tris-HCl, pH 7.0, containing 0.5 M NaCl, and finally with 100 ml 50 mM Tris-HCl, pH 7.0, with 0.2 M NaCl, the anti-LxS-MYB305 antibodies were eluted with 100 mM glycine, pH 2.5. The antibodies were neutralized with 1 M Tris-HCl, pH 8.5, 50% glycerol, 0.25 M NaCl, and 0.1 M KCl prior to storage at −20°C and use in the immunoblot experiments.

Phylogenetic Analysis

All sequences other than LxS-MYB305 were obtained from GenBank. The R2R3 MYB region of LxS-MYB305 was aligned with other MYB proteins’ MYB regions using the ClustalW integrated in the MEGA program (Tamura et al., 2007). The aligned sequences (see Supplemental Figure 5 and Supplemental Data Set 1 online) were used to produce phylogenetic trees using MEGA (Tamura et al., 2007). The phylogenetic trees were produced using the neighbor joining method with the following parameters: complete deletion and F-distance. One thousand bootstrap replications of each tree were produced to test the robustness of the phylogenetic tree. Similarly, the full-length LxS-MYB305 protein sequence and closely related protein sequences were aligned (see Supplemental Figures 6 and 7 and Supplemental Data Sets 2 and 3 online) and were analyzed using the same method and parameters as above.

Secondary Structure Prediction

The activation domain of the LxS-MYB305 coding sequence was identified from the one-hybrid studies and input on the Jpred server (www.compbio.dundee.ac.uk/www-jpred/index.html).

Expression of MYB305 in Escherichia coli and Purification of Recombinant Protein

The full-length cDNA of LxS-myb305 was amplified by PCR using the MYBGST1 and MYBGST2 primers (see Supplemental Table 1 online). The PCR product was digested with BamHI and SalI (Sigma-Aldrich) and ligated into the similarly digested vector pGEX-4T-3 (GE Life Sciences) to generate the expression vector, pRT609. The plasmid was then transferred into E. coli strain BL21 Codon Plus. The E. coli were grown in 2YT media with 1% glucose at 30°C, and 0.1% IP Isopropyl β-D-1-thiogalactopyranoside was used to induce the expression of the soluble fusion protein. The fusion GST-MYB305 protein was purified using Glutathione Sepharose 4B (GE Life Sciences). The protein concentration was determined by the method of Bradford (1976).

Preparation of Labeled Oligonucleotide Probes and Mobility Shift Assays

The probes for analysis of the MYB binding sites in the nec1 promoter were prepared by [32P]-labeling. The duplex N1S1 probe was prepared by annealing two single-stranded oligonucleotides, N1P1-UPPER and N1P1-LOWER (see Supplemental Table 1 online). The two oligonucleotides were mixed in a 0.2-mL tube and heated to 95°C using a thermal cycler. Then, the tube was floated on a 250-mL beaker filled with 95°C water sitting at room temperature. As the water temperature decreased and reached room temperature, the paired oligonucleotides annealed. Likewise, the duplex N1S0 probe was similarly prepared using the oligonucleotides N1P2-UPPER and NIP2-LOWER. Both probes were labeled with γ-[32P]ATP by T4 polynucleotide kinase (Promega). The duplex competitor probes N1C1 and N1C0 were similarly prepared. Mobility gel shift assays were performed using the Promega gel shift assay system according to the manufacturer’s instructions. The N5P probe for the analysis of the MYB binding site in the nec5 promoter was prepared using a fluorescent HEX tag incorporated during synthesis at the 5’ end of the oligonucleotide (Integrated DNA Technologies). The gel shift assay was performed the same way as with N1S1 and N1S0 probes, except that the gel was imaged with a Typhoon 8600 imaging system (GE Life Sciences) instead of x-ray film.

Transcriptional Activation Assays

Yeast One- and Two-Hybrid Assays

Details of the construct preparation for the yeast one- and two-hybrid studies are presented in the Supplemental Methods online. The constructs were transformed into the Saccharomyces cerevisiae strain MalV203 (Invitrogen) using the method of Gietz et al. (1992). The transformed yeast was cultured in complete synthetic yeast medium (Sherman et al., 1986) without Leu (SYM Leu−) for transactivation assays or in SYM Leu− Trp− for two-hybrid assays. In both cases, cells were grown in the liquid medium until they reached an OD600 of 1.0, before transferring to SYM Ura− or SYM His− plates for serial dilution growth assays.

β-Galactosidase Activity and Yeast Cell Growth Assays

β-Galactosidase activity in the transformed yeast cells was quantified using chlorophenol red-β-D-galactopyranoside (Sigma-Aldrich) as substrate (Serebriiskii and Golemis, 2000). The serial dilution growth of transformed yeast cells was assayed in SYM Leu− according to the manufacturer’s protocol (Invitrogen).

Subcellular Localization

Details of the construct preparation for the subcellular localization studies are presented in the Supplemental Methods online.

Protoplast Preparation and Transformation

In vitro shoot cultures of N. tabacum cv Xanthi were used for protoplast isolation. Plants were maintained on Murashige and Skoog (MS) solid medium (Murashige and Skoog, 1962) without growth regulators at 25°C. Sterile leaves from 2-month-old in vitro–grown plants were digested
overnight a solution of 1% (w/v) Onozuka Cellulase (Research Products International) and 0.2% (w/v) Macerozyme (Julie and Brent, 1986) dissolved in K3S medium (Nagy and Maliga, 1976). Following digestion, the protoplasts were collected and concentrated by floating on K3S1 medium in Bobcock bottles (Julie and Brent, 1986). The protoplasts were transiently transformed using polyethylene glycol (Locatelli et al., 2003). The transformed protoplasts were grown in K3G1 medium for 24 h without selection and then for a second 24-h period in K3G1 medium + 25 μg/mL Hygromycin B before observation.

**Fluorescence Microscopy**

Protoplasts selected as described above were imaged using an Olympus BX60 upright fluorescent microscope fitted with a Burner module (U-ULS100HG). Photographs were taken by Nikon Coolpix 950 digital camera using the GFP fluorescence filter. For each transformant, there were ~5 to 20 observations, and the cells shown are typical of each transformant.

**nec1 Promoter Assays**

Details of the construct preparation for the nec1 promoter assays are presented in the Supplemental Methods online.

**Plant Transformation**

*N. tabacum* cv Xanthi plants were transformed with *Agrobacterium tumefaciens* LBA 4404 (Ooms and Bakker, 1982), by cocultivation as described (Thornburg et al., 1987). Transgenic progeny lines were selected on MS plates containing 0.1 g/mL benzylaminopurine, 50 μM Hygromycin B, and 100 μg/mL Carbenicillin. After regeneration, plantlets were transferred to MS media with 50 μg/mL Hygromycin B and 100 μg/mL Carbenicillin, but containing no hormones for rooting. Once the rooted shoots were ~5 cm tall, they were transferred into soil and grown to floral maturity in the greenhouse.

**GUS Activity Assays**

Floral nectaries from the transformed tobacco plants were collected and the tissue was homogenized in extraction buffer (50 mM NaHPO4, pH 7.0, containing 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% [w/v] sodium lauryl sarcosine, and 0.1% [w/v] Triton X-100). The protein concentration was determined using the method of Bradford (1976). The activities of GUS were determined by fluorometric assays using 4-methylumbelliferyl-β-glucuronide as substrate (Jefferson, 1987).

**LxS-MYB305 Overexpression and LxS-MYB305 RNAi Plants**

The full-length LxS-myb305 cDNA sequence (AF132671) was PCR amplified by attBMYB-1 and attBMYB-2 primers and then recombined (BP reaction) into the pDONRZeo vector (Invitrogen) to obtain the entry clone pRT671. This plasmid was subsequently recombined (LR reaction) into pMD19-1 (Invitrogen) to obtain the overexpression plant transformation vector, pRT672. Transgenic plants were prepared as described above.

The 3’ end of the cDNA sequence of LxS-myb305 excluding MYB DNA binding domains was PCR amplified by attBMYB-13 and attBMYB-2 primers and subsequently recombined (BP reaction) into pDONRZeo to obtain the entry clone, pRT677. This plasmid was then recombined (LR reaction) into pB7GWIWG2 (Karimi et al., 2002) to get an RNAi plant transformation vector, pRT672. Transgenic plants were prepared as described above, except the RNAi plants were selected by 20 μg/mL ammonium glutosinate instead of Hygromycin B in both selection and rooting stages.

**RT-PCR**

The tobacco tissues were collected and frozen in liquid nitrogen; RNAs were then purified using the TRizol reagent method (Invitrogen) according to the manufacturer’s instructions. The first-strand cDNAs were made using the Eppendorf cMaster RT-PCR system. All primers used in this study are given in Supplemental Table 1 online. Minimal cycles of amplification were used to detect the genes of interest to ensure that analyses were conducted in a linear portion of the amplification spectrum. Initially, RT-PCR reactions were run with even cycle numbers starting from 20 cycles on. After analysis, the cycle number when the bands first became visible was chosen for the final analysis conditions. The gels were stained with ethidium bromide, and individual cycle numbers are indicated in each figure legend.

**Quantitative RT-PCR**

The leaves of LxS-MYB305 overexpression *N. tabacum* and nectaries of the LxS-MYB305 RNAi *N. tabacum* plants were collected and frozen in liquid nitrogen. Total RNA was purified using the TRizol reagent method according to the manufacturer’s instructions. The first-strand cDNAs were made using the Eppendorf cMaster RT-PCR system, and the first-strand cDNAs were used as template in the quantitative PCR reaction. Primers used are given in Supplemental Table 1 online. The PCR reactions were performed on Stratagene mx4000 multiplex quantitative PCR system using the Brilliant II SYBR Green QPCR Master Mix (Stratagene). 26S rRNA was used as an internal reference to normalize the relative level of each transcript. Four to six biological replications were used to calculate each relative expression value.

**Immunoblotting**

Various tobacco tissues were collected and immediately frozen in liquid nitrogen. Proteins were isolated using a plant total protein extraction kit (PE0230; Sigma-Aldrich), and 100 μg of total protein from leaves or different nectary stages were electrophoresed on 12% SDS-PAGE. After running the gel, the proteins were electrophoretically transferred to polyvinylidene fluoride membranes. The proteins were detected with either 1:2000 rabbit anti-LxS-MYB305 antiserum or 1:5000 rabbit anti-NEC1 antiserum and then with horseradish peroxidase-conjugated goat anti-rabbit antiserum. The SuperSignal West Pico Chemiluminescent Substrate (Pierce Scientific) was used to detect the signals on membranes; the membranes were then exposed to x-ray films. Individual exposure times are indicated in each figure legend.

**Accession Numbers**

The sequence of the LxS8-MYB305 cDNA used in these studies was deposited in GenBank with accession number EU111679. A second, nearly identical MYB305 cDNA was also cloned and was deposited with accession number EU111678. Other genes used in this study include the promoters of the *Nicotiana plumbaginifolia nec1* gene (AF132671) and nec5 gene (AF503441). Sequences used in the phylogenetic analysis are presented in the legends of the supplemental figures online.

**Supplemental Data**

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

**Supplemental Figure 1.** Yeast Two-Hybrid Test of Homodimerization with LxS-MYB305.

**Supplemental Figure 2.** Comparison of Wild-Type and MYB305OX Phenotypes.

**Supplemental Figure 3.** Gene Expression in Wild Type and Mutant Plants.
Supplemental Figure 4. Phylogenetic Analysis of Lxs-MYB305 with Rosid Homologs.

Supplemental Figure 5. Alignment of R2R3 MYB DNA Binding Domains for Phylogenetic Analysis.

Supplemental Figure 6. Alignment of Complete MYB Amino Acid Sequences for Phylogenetic Analysis.

Supplemental Table 1. Oligonucleotides Used in These Studies.

Supplemental Table 2. Length of Various Organs from Flowers at Anthesis.

Supplemental Data Set 1. Alignments Used for the Phylogenetic Analysis in Figure 2A.

Supplemental Data Set 2. Alignment Used for the Phylogenetic Analysis in Figure 2B.

Supplemental Data Set 3. Alignment Used in Supplemental Figure 4.

Supplemental Methods. Preparation of DNA Constructs for Experiments Used in These Studies.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation (IBN 0235645), Carver Trust, the Hatch Act, and State of Iowa funds.

Received April 15, 2008; revised July 28, 2009; accepted August 26, 2009; published September 25, 2009.

REFERENCES


Li, J., Yang, X., Wang, Y., Li, X., Gao, Z., Pei, M., Chen, Z., Qu, L.J.,...


The MYB305 Transcription Factor Regulates Expression of Nectarin Genes in the Ornamental Tobacco Floral Nectary
Guangyu Liu, Gang Ren, Adel Guirgis and Robert W. Thornburg
Plant Cell 2009;21;2672-2687; originally published online September 25, 2009;
DOI 10.1105/tpc.108.060079

This information is current as of July 5, 2017

Supplemental Data /content/suppl/2009/09/16/tpc.108.060079.DC1.html
References This article cites 61 articles, 17 of which can be accessed free at:
/content/21/9/2672.full.html#ref-list-1
eTOCs Sign up for eTOCs at:
http://www.plantcell.org/cgi/alerts/ctmain
CiteTrack Alerts Sign up for CiteTrack Alerts at:
http://www.plantcell.org/cgi/alerts/ctmain
Subscription Information Subscription Information for The Plant Cell and Plant Physiology is available at:
http://www.aspb.org/publications/subscriptions.cfm

© American Society of Plant Biologists
ADVANCING THE SCIENCE OF PLANT BIOLOGY