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Functional Study of Transcription Factor MYB305 in Tobacco Flower Development

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Functional study of transcription factor MYB305 in tobacco flower development

by

Guangyu Liu

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Molecular Cellular and Development Biology

Program of Study Committee:
Robert Thornburg, Major Professor
  Martha James
  Basil Nikolau
  Thomas Peterson
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Iowa State University
Ames, Iowa
2010

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Abstract

We isolated and characterized the cDNA encoding the ornamental tobacco (N.langsdorffii X N.sanderae) homolog of the antirrhinum (Antirrhinum majus) MYB305. This cDNA encodes a MYB family transcription factor protein containing a conserved R2R3 MYB DNA binding domain. This myb305 gene is expressed uniquely in floral organs with the highest level in the mature nectary and with lower levels in the ovary, floral tube, petals and flower abscission zone. A GFP-MYB305 fusion protein localizes to nucleus of tobacco protoplasts and yeast one-hybrid assays demonstrates that it functions as a transcription activator. A conserved 23 amino acid C-terminal domain is required to activate gene expression. Functional study discovered that MYB305 is involved in many physiological processes in plants.

First, MYB305 regulates expression of the major nectarin genes Nectarin I (nec1) and Nectarin V (nec5) in the nectary of ornamental tobacco plants. Temporally, myb305 gene expression precedes that of nec1 and nec5 genes. The purified GST-MYB305 proteins bind two consensus MYB-binding sites on the ornamental tobacco nec1 promoter as well as bind the single site located on the nec5 promoter. Deletions of either of the binding sites from the nec1 promoter significantly reduce expression in nectary tissues. Ectopic expression of MYB305 in foliage is able to induce the expression of both nec1 and nec5. Further knockdown of MYB305 result in reduced expression of nec1 and nec5.

Second, MYB305 plays important roles in floral organ development and maturation by disrupting starch metabolism. In the MYB305 knockdown plants, the nectaries retain juvenile character and secrete reduced levels of nectar, the petals are smaller and do not fully expanded during anthesis. Abnormal starch metabolism has been found to contribute to these phenotypes because reduced starch accumulation was found in both the nectary, before nectar secretion, and in petals, before anthesis. The reduced levels of starch accumulation correlate with the expression of starch metabolic genes in both nectary and petal of MYB305 RNAi plants, suggesting that MYB305 is involved in starch metabolism by controlling the expression of starch metabolic genes.
Chapter 1  General Introduction

1.1 Flower, nectar and nectary

Flowers, also known as blooms or blossoms, are the reproductive organs found in flowering plants or angiosperms (Darwin, 1890). The biological function of a flower is to produce seeds through sexual reproduction and thus to complete the plants’ life cycle (Friis, 1990). This process begins with pollination, is followed by fertilization and results in the formation and dispersal of the seeds (Darwin, 1862; Peter, 2001). The seeds are the next generation and serve as the primary means by which individuals of a species are capable of regenerating as well as spreading into other landscapes (Friis, 1990).

The study of flowers began at ~12,000 years ago when human beings started to domesticate the wild plants for food (Smyth, 2005). However, due to the missing of historical evidence, the earliest historical records of description of flowers came from ~2300 years ago, written by the Greek philosopher Theophrastus (~370-285 BC), who defined that the flowers are composed of the petals and carpels (Smyth, 2005). After that, the morphology of the flowers has been studied for centuries. In the current opinions, the flowers are generally considered to have the following floral organs: sepal, flower tube and petal are on the outside; stigma, style, anther, filament, ovary and nectary are on the inside. The flowers with typical floral organs from \( N. \text{tabacum} \) cv Xanthi and ornamental tobacco (\( N. \text{langsdorffii} \times N. \text{sanderae} \), \( LxS8 \)) plants are shown on the Figure1.1.

Flowers are the reproductive organs of flowering plants, they accomplish the sexual reproduction process by mediating the joining of the sperm, contained within pollen, to the ovules, contained within the ovary (Endress, 1999; Etcheverry et al., 2008). Pollination is the movement of pollen from the anthers to the stigma. The joining of the sperm to the ovules is called fertilization. The fertilized ovules produce seeds that are the next generation (Lord and Russell, 2002).
above sexual reproduction steps, pollination is the most important and uncertain step. Flowering plants have two types of pollination strategy, abiotic pollination and biotic pollination. Abiotic pollination refers the pollination process which is mediated without the involvement of other organisms. The most common abiotic pollination is mediated by wind and this form of pollination is predominant in grasses, conifers and many deciduous trees. Another form of pollination is biotic pollination which requires the pollinators: organisms that carry or move the pollen grains from the anther to the pistil. About 80% of the flowering plants on earth are biotically pollinated (McGregor, 1976) and biotic pollination is perhaps the most common and reliable strategy for the flowering plants (Darwin, 1862).

Successful biotic pollination requires flowers to develop a suit of traits to attract pollinators. The traits include flower shape, size, color, scent, reward type and amount, nectar composition, timing, etc (Faegri, 1979; Proctor M 1996). Although flower shape, color and scent can temporarily attract the pollinators, sustainable biotic pollination requires mutually beneficial relationship between both flowers and pollinators: the pollinators get rewards from the flowers and the flowers get pollinated by the pollinators (Arnone, 1993; Schiestl, 2003). The typical rewards are pollen and nectar (Feinsinger, 1983; Neiland, 1998; Cresswell, 1999).

In view of evolution, the pollen was the initial reward that flowers offered to the pollinators and many existing species of pollinators still collect pollens when they visit flowers (Percival, 1961; Feinsinger, 1983; Temeles, 2003). However, the pollen is indigestible for many insects including most butterflies, which resulted in the sugar rich liquid, nectar, became a more widely used reward (Feinsinger, 1983; Temeles, 2003). Nectar contains small molecules, like sugars, amino acids, lipids, organic and inorganic acids, glycosides, ions, aromatic compounds, as well as large molecules, such as proteins and pigments (Inouye, 1983; Pleasants, 1983; Wunnachit, 1992; Davis, 1998; Galetto, 2004). The major sugars in floral nectar are sucrose and its two monosaccharide components, glucose and fructose (Davis, 1998; Pacini, 2003).
Figure 1.1 Structure of tobacco flowers.
Panel A (left) *N.tabacum* cv Xanthi flower
Panel B (right) *LxS8* ornamental tobacco flower
Nectar is secreted by nectariferous tissues of plants. These tissues can be classified into two major classes: extrafloral nectary and floral nectary (Durkee, 1981; Schmid, 1988; Vogel, 1997, 1998a, 1998b, 1998c). The extrafloral nectaries are often found on outer floral parts or on vegetative parts and perform photosynthesis (Heads, 1985). Because they lack direct vascular tissues, the extrafloral nectaries of angiosperms usually grow near vascular bundles and are frequently associated with developing organs, which make the fluids in the vascular system easily accessible to them (Durkee, 1981; Nepi, 1996). Floral nectaries are typically embedded in the floral tube, secreting nectar to attract pollinators to access the flowers. The floral nectaries are often composed of three types of structure components: epidermal cells, parenchyma cells, and vascular bundles in some flowers (Vogel, 1997, 1998a, 1998b, 1998c). The epidermal cells are responsible for secretion of carbohydrate and amino acids to nectar, the parenchyma cells store polysaccharides, and vascular cells are involved in fluid and nutrients transportation. However, floral nectaries of angiosperms have many different anatomy structures that vary from species to species, such as position in flowers, nectary structures, and types of plastids (Vogel, 1997, 1998a, 1998b, 1998c). The tobacco floral nectary gland is located at the base of floral tube (Figure 1.1) and it secretes a large amount of nectar at late stage of floral development.

1.2 Nectar proteins (nectarins) and nectar redox cycle

Floral nectar contains many proteins that are essential for plant defense and survival. In the nectar of carnivourous plants, such as albany pitcher plant *Cephalotus follicularis* and lobster-pot plant *Nepenthes aristolochioides*, the commonly found proteins are proteases and acid phosphatases which help plants to digest the trapped preys to feed themselves (Darwin, 1875; Heslop-Harrison, 1975, 1976; Juniper, 1989; Plachno, 2005). In the nectar of leek plant *Allium porrum* L., high levels of lectin and alliinases are found to be involved in plant defense reaction (Peumans et al., 1997). Among all identified the nectar proteins,
the best studied nectar proteins come from the nectar of ornamental tobacco plants and we will review these nectar proteins in terms of their functions.

The proteins that are secreted into nectar of the ornamental tobacco plant are termed nectarins. These nectarins accumulate to up to 250μg/ml in nectar and the SDS-page analysis revealed that there are five nectarin proteins in ornamental tobacco nectar (Figure 1.2 A) (Carter and Thornburg, 2004a). These proteins were named Nectarin I (NEC1) through Nectarin V (NEC5) in order of increasing molecular weight.

Nectarin I (NEC1) is the most abundant protein in the nectar of ornamental tobacco. NEC1 represents ~50% of total nectar proteins and its molecular weight is 24.6 kDa for pre-mature protein and 21 kDa for matured protein in the nectar. The expression of NEC1 is limited in the nectary glands and is developmentally regulated (Carter and Thornburg, 2000b; Carter and Thornburg, 2004a). It is only expressed when the nectar is being actively secreted from the nectary (Carter and Thornburg, 2000b). The bioinformatics and biochemistry studies have shown that the NEC1 protein is a germin-like protein (GLPs) and it functions as a superoxide dismutase which removes superoxide while generating hydrogen peroxide (Figure 1.2 B) (Carter and Thornburg, 2000b).

Nectarin II (NEC2; 35 kDa) is a breakdown product of Nectarin III (NEC3; 40 kDa) and both NEC2 and NEC3 proteins share identity with dioscorin, the major soluble protein of yam tubers (Conlan et al., 1995; Carter and Thornburg, 2004d). NEC3 is a bifunctional enzyme with monodehydroascorbate reductase and carbonic anhydrase activities. It can catalyze the production of bicarbonate and can convert monodehydroascorbate into ascorbate. The function of NEC3 is to stabilize the pH of nectar and to maintain the ascorbate concentrations in nectar (Carter and Thornburg, 2004d).

Nectarin IV (NEC4; 60 kDa) is an endoglucanase inhibitor and its expression is limited in the nectary gland of ornamental tobacco at post-fertilization stage.
A. SDS PAGE of ornamental tobacco nectar proteins

B. Nectar Redox Cycle

C. NEC4 is a fungal endoglucanase inhibitor

Figure 1.2 Nectaries of ornamental tobacco
Panel A  SDS PAGE separation of nectar proteins
Panel B  Redox Cycle in the nectar of ornamental tobacco plants
Panel C  NEC4 protein inhibits fungal endoglucanase
(Naqvi et al., 2005b). The function of NEC4 is to inhibit the fungal proteins that degrade plant cell walls (Naqvi et al., 2005a).

Nectarin V (NEC5) is the largest soluble protein in ornamental tobacco nectar. Its expression is very similar to the expression of NEC1, which is limited in the nectary gland at late development flower stages. It is a flavin-containing berberine bridge enzyme (BBE)-like protein and possesses glucose oxidase activity (Carter and Thornburg, 2004b). NEC5 can convert the glucose in the nectar into gluconic acid with the production of hydrogen peroxide (Carter and Thornburg, 2004b).

Studies show that all of the above nectarines are involved in defense reaction in the nectars. NEC4 itself is a fungal endoglucanase inhibitor which prevent the flower from being contaminated from the fungus. NEC1, NEC2/3 and NEC5 plus nectary NOX1 (NADPH oxidase 1) form a unique redox cycle which utilize high levels of H₂O₂ to defend nectar from being contaminated by microorganisms (Figure 1.2 B) (Thornburg et al., 2003; Carter and Thornburg, 2004a). In this redox cycle, the oxygen was converted into H₂O₂ via NOX1/NEC1 and NEC5, the concentration of H₂O₂ can be as high as 4mM and this dangerous high level of H₂O₂ is balanced by β-carotene and ascorbate-MDHA, regulated by NEC2/3 (Thornburg et al., 2003; Carter and Thornburg, 2004c).

1.3 Carbohydrates, nectar and nectary

Floral nectar is usually rich in carbohydrates (Percival, 1961; Wittich, 1998). These substances are thought as an important nutrients for attracting pollinators (Pyke, 1991; Fahn, 2000; Pacini, 2003). The major carbohydrates in nectar are sucrose and its two monosaccharide components, glucose and fructose (Vogel, 1997; Davis, 1998; Vogel, 1998a, 1998b, 1998c). Honeybee and insects can be attracted by highly concentrated sucrose-dominated nectar, while hummingbird can be attracted by monosaccharide-dominated nectar (Nepi, 1996; Pacini, 2003). The preferences of different pollinators result in the variety of nectar production rate and nectar composition (Baker, 1983; Pellmyr, 1992).
The nectar carbohydrates are secreted from nectaries and there are two types of floral nectaries in higher plants (Figueiredo, 1992; Nepi, 2001; Pacini, 2003). The first type is chloroplast nectary, where starch is synthesized and deposited in the chloroplasts during the day but degrades for nectar production at night. The second type is amyloplast nectary, where high levels of starch are accumulated in the nectary parenchyma cells during floral development and can be rapidly broken down to provide soluble sugars for nectar secretion. The ornamental tobacco nectary is amyloplast type nectary and quantification study of ornamental tobacco nectary starch revealed that there is very little starch at an early flower developmental stage, soon thereafter the amount of starch increased dramatically, reaching a peak approximately 24 hours prior to anthesis (Horner et al., 2007; Ren et al., 2007c). During the last 24 hours before anthesis, the amount of starch decreased rapidly, and the accumulated starch is converted to sugars (sucrose, glucose and fructose) for nectar production prior to anthesis (Ren, 2007).

Starch metabolisms in the nectary share core pathways with other organs in higher plants (Zeeman et al., 2002; Smith et al., 2005; Gould et al., 2008). These pathways are generally consisted by three specific groups of enzymes, the enzymes in starch biosynthesis pathway, the enzymes in starch degradation pathway and the enzymes involved in both starch biosynthesis and degradation pathways. These enzymes are highly conserved in all of the plant species. In general, the process of starch biosynthesis is initiated by the enzyme ADP-glucose pyrophosphorylase (AGP), which convert the phosphorylated glucose to the ADP-Glucose (Baroja-Fernandez et al., 2003). The ADP-Glucose can be added to the non-reducing end of a pre-existing starch glucan primer by either soluble starch synthases (SS) or granule bound starch synthases (GBSS) (Delvall et al., 2005). As the chain of starch continues growing, the branching of the starch can be introduced by starching branching enzyme (SBE) via a-1,6 glycosidic bond (Myers et al., 2000; Devillers et al., 2003), and the restructuring of the branch point can be regulated by starch de-branching enzymes (DBE) (Figure 1.3A) (Dinges et al., 2001; Dinges et al., 2003; Hussain et al., 2003). The newly synthesized starch is
Figure 1.3 The core pathway of starch biosynthesis and degradation in higher plants.
AGPase - ADP-glucose pyrophosphorylase; SS - Starch synthase; GBSS - Granule bound starch synthase; SBE - Starch branching enzyme; DBE - Starch debraching enzyme; SP - Starch phosphorylase; GWD - Glucan water dikinase; PWD - phosphoglucon water diskinase; DPE - Disproportionating enzymes.
usually stored in the form of insoluble granules in plastids or chloroplasts of plants. The starch granule has semicrystal structure and starch degradation begins with the breakdown of the long starch molecules by endoamylases (α-amylase, AAM, in cereal) at the surface of the granule. With the help from glucan water dikinase (GWD) and phosphoglucan water dikinase (PWD), the endoamylases catalyze the reaction to convert starch to either linear glucans or branched glucans (Smith et al., 2005). The branched glucans can be degraded to linear glucans by DBE. Then, the soluble linear glucans are cut into the small molecules by β-amylase (BAM), disproportioning enzymes (DPE) and α-glucosidase (Figure 1.3B). The starch synthesis and degradation are developmentally controlled in higher plants. In ornamental tobacco nectary, it has been shown that the starch synthesis genes and degradation genes occur at different times. Starch accumulates at early stages before anthesis in the nectary of tobacco. Most starch anabolic genes, including AGP and SS, are expressed in early stages, and are down regulated at the late stages. In contrast, starch catabolic genes, such as isoamylase (ISA), AAM and BAM, are not expressed at early stages, but are robustly expressed at late floral stages (Ren, 2007; Ren et al., 2007b). As a result, the starch accumulated in early stages is rapidly degraded to provide soluble carbohydrates for nectar secretion at late stages before anthesis.

1.4 MYB transcription factors and their functions in plants

The plant growth and development are regulated by coordination of many classes of genes, such as the genes affecting hormone metabolism, signaling and cell cycle (Victor et al., 2008). Among these genes, transcription factors are one of the best known classes of genes involved in controlling the flower development (Medard and Yanofsky, 2000; Dinneny and Yanofsky, 2004). Transcription factors are important regulators of gene expression and they are composed of at least two domains, DNA binding domain and transcription activation domain. They have been categorized into different families based on the conserved DNA binding domain, such as helix-loop-helix, leucine zipper, zinc finger and MADS cassette.
Figure 1.4 Structure of MYBs
Panel A. Primary structure of three types of plant MYB proteins
Panel B. Interaction between R1R2R3 type MYB protein and dsDNA
Among these transcription factor families, the MYB genes form the largest transcription factor gene family in plants (Stracke et al., 2001).

The first MYB gene identified was the oncogene \(v\)-MYB (\(v\)irus induced \(y\)eloBlastosis, MYB) from the avian virus. Later, many \(v\)-MYB related genes were found in vertebrates (\(c\)-MYB, \(A\)-MYB and \(B\)-MYB) as well as other species, including insects, plants and fungi (Martin and Paz-Ares, 1997). These MYBs have been found to be involved in the regulation of cell proliferation, differentiation, and apoptosis (Weston, 1998). The first plant MYB gene was found in maize, which is a \(c\)-MYB like protein involved in anthocyanin biosynthesis (Paz-Ares, 1987). Later, large numbers of MYB proteins were found in plants to be involved in diverse physiological and biochemical processes including the regulation of secondary metabolism, control of cell morphogenesis, regulation meristem formation, floral and seed development, and control of the cell cycle (Jackson et al., 1991; Sablowski et al., 1994; Gubler et al., 1995; Moyano et al., 1996; Abe et al., 1997; Martin and Paz-Ares, 1997; Wang et al., 1997; Penfield et al., 2001).

All MYB proteins contain a MYB DNA binding domain which is conserved amongst animals, plants and yeasts (Lipsick, 1996). This MYB DNA binding domain usually consists of one to three imperfect repeats (R1, R2, and R3) (figure 1.4A). Each repeat is around 51 amino acids long and encodes three alpha-helices forming a helix-turn-helix (HTH) structure which can interact with the major groove of double strand DNA (Figure 1.5). Because of this special structure, the MYB proteins are categorized into three groups according to the repeats presented in the MYB DNA binding domain, they are R1R2R3-MYB, R2R3-MYB and R1-MYB. Most plant MYB proteins are R2R3-MYBs and a majority of them can recognize the MBSIIG binding site ([T/C]ACC[A/T]A[C/A]C) on many of their target genes (Romero et al., 1998; Stracke et al., 2001).

In higher plants, the large number of MYB-related genes results in the diversity of their functions in plant growth and development. For example, several MYB proteins, including TTG1, GL1, GL2, WER and CPC, are involved in trichome
cell fate determination (Herman and Marks, 1989; Oppenheimer et al., 1991; Di Cristina Sessa, 1996; Wada et al., 1997); Arabidopsis At-MYB2 can be transiently induced by dehydration, high-salt stress and ABA induction, which affect the adaptation of vegetative tissues to abiotic environmental stresses (Abe et al., 1997; Abe et al., 2003); At-MYB68 is regulated by temperature, and the At-MYB68 knockdown plants decrease the grow rate in high temperature (Feng et al., 2004); GAMYB was found in the GA signaling pathway and it is able to induce the expression of GA-inducible genes by interacting with the GA-responsive cis-elements of the these genes in aleurone tissue (Frank et al., 1999; Gocal et al., 2001); Am-MYB305 and Am-MYB340 redundantly control the expression of the gene encoding the first enzyme of phenylpropanoid metabolism, phenylalanine ammonia-lyase (PAL), and the gene encoding the enzymes in flavonoid synthesis pathway (Moyano et al., 1996).

Based on their functions, MYB proteins can be classified into four classes: control of cell morphogenesis, responses to environmental stress, response to phytohormone and involve in the secondary metabolism pathways (Du et al., 2009). And more functions of MYB transcription factors are waiting to be discovered.

1.5 Outline of this dissertation

We have previously determined that a MYB protein is responsible for transcriptional regulation of nec1 gene expression in the nectary of LxS8 (N.langsdorffii X N.sanderae) ornamental tobacco (Carter and Thornburg, 2003b). Later, a myb cDNA that is robustly expressed in the nectary of LxS8 plants was cloned as part of a nectary EST project that analyzed 3 different stages of nectary development [Stage 6 (S6), presecretory; S12, secretory at anthesis; and PF = post-fertilization, senescent] (Yin et al., unpublished). Because this ornamental tobacco MYB protein is homologous to antirrhinum majus MYB305, we named this MYB protein (LxS) MYB305.
We first characterized this MYB305 in detail (Chapter 2). This myb305 gene is expressed uniquely in flowers and its MYB305 protein is homologous to several MYB proteins that are found actively expressed in the flowers of other species. The MYB305 protein localizes to the nucleus of tobacco protoplasts and is a transcription activator in yeast. A conserved transcription activation domain is found on the C-terminal of MYB305 protein and the acidic residues on this domain mediate transcription activation. Then, we study the relationship between MYB305 and Nectarin genes (Chapter 2). The purified MYB305 binds to the MYB binding sites on the nec1 and nec5 gene promoter, and these MYB binding sites are required for normal transcriptional activation of the nec1 gene. The myb305 gene is expressed before nec1 and nec5 genes, overexpression of MYB305 leads to increased expression of nec1 and nec5, knockdown of MYB305 results in decreased expression of nec1 and nec5. In Chapter 3, we observed additional phenotypes in the MYB305 knockdown plants. The nectary shows retention of juvenile character and petals fails to expand fully during anthesis in the MYB305 knockdown plants. These phenotypes are associated with disrupted starch metabolism and correlated with changed expression pattern of starch metabolic genes. Finally, we summarized our findings in Chapter 4 and present some preliminary work for future study.
Chapter 2  The MYB305 transcription factor regulates expression of *Nectarin* genes in the ornamental tobacco floral nectary

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Abstract

We have isolated and characterized the cDNA encoding the ornamental tobacco (*Nicotiana langsdorffii X Nicotiana sanderae*) homolog of the antirrhinum (*Antirrhinum majus*) MYB305. The ornamental tobacco MYB305 was robustly expressed at Stage 12 of nectaries but was only weakly expressed in the earlier Stage 6 nectaries. This MYB305 contains a conserved R2R3 MYB DNA binding domain with 76 amino acids in the activation domain. A GFP-MYB305 fusion protein 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 *E.coli* as a GST 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 the expression of both *nec1* and *nec5*, as well as two flavonoid biosynthetic genes in the foliage. Finally RNAi knockdown of MYB305 resulted in reduced expression of *nec1, nec5, pal*

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and chi. We conclude that expression of MYB305 regulates expression of the major nectarin genes in the floral nectary.

2.1 Introduction

Many species of angiosperms attract animals (insects, birds, 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 nectars 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 Nicotiana sanderae*) plants (Carter et al., 1999; Carter and Thornburg, 2000a; Carter and Thornburg, 2004d; Naqvi et al., 2005b). 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 we propose has
evolved to defend the gynoecium from invading microorganisms (Thornburg et al., 2003; Carter and Thornburg, 2004a).

The Nectar Redox Cycle involves the production of very high levels of hydrogen peroxide: up to 4 mM (Carter and Thornburg, 2000a). This pathway is initiated by a developmentally regulated, nectary-expressed NADPH oxidase that begins producing superoxide just prior to anthesis (Carter et al., 2007b). The superoxide is subsequently disproportionated into oxygen and hydrogen peroxide by the major nectar protein, Nectarin I (NEC1) (Carter and Thornburg, 2000a). 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, 2004b). Because of the highly oxidative nature of ornamental tobacco nectar, antioxidants (both ascorbate and Beta-carotene) are important in nectary function (Carter and Thornburg, 2004d; Horner 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., 2005b).

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 and leek, have been completely characterized. In leek, a mannose binding protein and alliinase were identified (Peumans et al., 1997) and both of these proteins are thought to function in defense of nectar. Recently, nectarins have been identified in extrafloral nectar of *Acacia spp.* in *Jacaranda mimosafolia* floral nectar, as well as in the reproductive secretions of gymnosperms. In each of these cases, the defensive proteins found in extrafloral nectar (Heil et al., 2005; González-Teuber et al., submitted), in Jacaranda floral nectar (Kram et al., 2008), and in gymnosperm secretions (Poulis et al., 2005; O'Leary et al., 2007; Wagner et al., 2007) appear to be classical defense proteins such as pathogenesis related proteins lipase, and thaumatin. Thus, nectarins represent a unique and quite varied group of proteins that function to protect plant secretions using a variety of molecular strategies.
To better understand the mechanisms that regulate expression of the nectarins in ornamental tobacco nectar, we have analyzed nectarin gene expression and determined that several different transcriptional programs regulate their expression (Carter et al., 1999; Carter and Thornburg, 2004d; Naqvi et al., 2005b). For Nectarin I (NEC1), the major nectar protein, we previously evaluated the expression from the nec1 promoter in transgenic plants (Carter and Thornburg, 2003b). 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, 2003b). This consensus MYB binding site was previously identified in flavonoid biosynthetic genes, including phenylalanine ammonia lyase (pal) and chalcone isomerase (chi) of antirrhinum, bean and parsley (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 Antirrhinum 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 (Yin and Thornburg, unpublished results). One outcome of this study was the identification of a highly-expressed MYB transcription factor in the nectary. This current study was, therefore, designed to investigate a potential role of this highly-expressed MYB transcription factor in regulating nec1 and nec5 expression.

2.2 Materials and methods
2.2.1 Materials

Unless otherwise noted, the chemicals used in these studies were obtained from either Fisher Chemical Co (www.fishersci.com) or Sigma Chemical Co (www.sigmaaldrich.com) and were of the highest quality available. All radioisotopes were from Perkin Elmer Corp (las.perkinelmer.com).
2.2.2 Plants

The LxS8 ornamental tobacco plants (*Nicotiana tabacum*) used in these studies, the growth condition of plants and methods for isolation of nectar and floral tissues was previously described (Carter et al., 1999). If not used immediately, tissues were frozen at -20°C until use. Flowers were staged as described (Koltunow et al., 1990). *Nicotiana 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 Dr. John Browse, Washington State University.

2.2.3 Genes and accession numbers

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, 2004d) (Naqvi et al., 2005b). The sequence of the LxS8-myb305 cDNAs were deposited in GenBank with accession numbers EU111678 and EU111679. Other genes used in this study include the promoters of the *N. plumbaginifolia* nec1 gene (AF132671) and nec5 gene (AF503441). Sequences used in the phylogenetic analysis are presented in the legends of those figures.

2.2.4 Phylogenetic Analysis

All sequences other than LxS-MYB305 were obtained from GenBank (www.ncbi.nlm.nih.gov). The R2R3 MYB region of LxS-MYB305 was aligned with other MYB proteins’ MYB regions using the ClustalW integrated in the MEGA program (Tamura K, 2007). The aligned sequences (Appendix B) were used to produce phylogenetic trees using MEGA (Tamura K, 2007). The phylogenetic trees were produced using the neighbor-joining method with the following parameters: complete deletion and P-distance. 1000 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 (Appendix C), and were analyzed by using the same method and parameters as above.

2.2.5 Subcellular localization

Constructs

The full-length LxS-myb305 gene coding region was PCR amplified from LxS-myb305 gene (Accession # EU111679) using primers attBMYB-1 and attBMYB-2 (Appendix A). The PCR product was gel-purified and cloned into pDONR/ZEO vector (www.invitrogen.com) to generate entry vector pRT613 containing the full length LxS-myb305 coding region. The pRT613 was recombined into the Curtis vector pMDC43 (Curtis and Grossniklaus, 2003) to generate the destination vector pRT656, which contained the gfp-myb305 gene fusion. Similarly, the truncated LxS-myb305 coding sequences were obtained by using different primers. Primer attBMYB-1 and attBMYB-4 were used to generate entry clone pRT636; attBMYB-3 and attBMYB-2 were used to generate entry clone pRT637; the vectors pRT636 and pRT637 containing deletions within the LxS-myb305 coding sequence were recombined into pMDC43 to generate vectors pRT657 and pRT658, respectively.

Protoplast preparation and transformation

In vitro shoot cultures of N. tabacum cv Xanthi were used for protoplast isolation. Plants were maintained on 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 (www.rpicorp.com) and 0.2% w/v Macerocyme (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 PEG (Locatelli et al., 2003). The transformed protoplasts were grown in K3G1 medium for 24h without selection then for a second 24h period in K3G1 medium + 25ug/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 about 5 to 20 observations and the cells shown are typical of each transformant.

2.2.6 Transcriptional activation assays and yeast two-hybrid assay Constructs

Initially, the full-length LxS-myb305 coding region was PCR amplified from LxS-myb305 gene (Accession # EU111679) using primers attBMYB-1 and attBMYB-2 (Appendix A). The PCR product was gel-purified and cloned into pDONR/ZEO vector (www.invitrogen.com) to generate entry vector pRT613. The entry clone pRT613 was then recombined into pDEST22 and pDEST32 (www.invitrogen.com) using LR clonase to generate pRT623 and pRT624 containing the GAL4AD and GAL4DB in frame with the full length LxS-myb305 respectively. Similarly, truncated LxS-myb305 fragments were cloned into pDONR/ZEO and subsequently were recombined into pDEST32. Primers used to generate these various truncated clones (Appendix A) are as follows: attBMYB-1 and attBMYB-4 were used to generate entry clone pRT636 (and the destination clone pRT631); attBMYB-3 and attBMYB-2 were used to generate entry clone pRT637 (pRT632); attBMYB-5 and attBMYB-2 were used to generate entry clone pRT638 (pRT633); attBMYB-7 and attBMYB-2 were used to generate entry clone pRT639 and (pRT634); attBMYB-1 and attBMYB-6 were used to generate entry clone pRT640 and (pRT635).

To generate activation domain mutated construct pRT648, a synthesized single strand DNA, MYBT-S3, was used as a template to PCR with primer attBMYB-7 and attBMYB-2, the PCR product was cloned into pDONR/ZEO (to get entry clone pRT643) and then into pDEST32 (to generate pRT648). Likewise, we used MYBT-S4 and MYBT-S5 as templates with primers attBMYB7 and
attBMYB12 to generate entry clone pRT651 and pRT652 and subsequent destination clone pRT653 and pRT654 respectively.

β-Galactosidase activity and yeast cell growth assays

The constructs were transformed into the *S. cerevisiae* strain MaV203 (www.invitrogen.com) using the method of Geitz et al (Gietz et al., 1992). The transformed yeast were cultured in complete synthetic yeast medium (Sherman et al., 1986) without leucine (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 in the transformed yeast cells was quantified by using chlorophenol red-β-D-galactopyranoside (CPRG) (www.sigmaaldrich.com) 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 (www.invitrogen.com).

### 2.2.7 EMSA (Electro Mobility Shift Assay, Gel Shift Assay)

**Expression of LxS-MYB305 protein in Escherichia coli and purification of recombinant protein**

The full-length cDNA of *LxS-myb305* gene was amplified by PCR using the MYBGST1 and MYBGST2 primers (Appendix A). The PCR product was digested with *Bam*HI and *Sal*I (Sigma) and ligated into the similarly digested vector pGEX-4T-3 (www.gelifesciences.com) to generate the expression vector, pRT608. 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% Isopropyl β-D-1 thiogalactopyranoside (IPTG) was used to induce the expression of the soluble fusion protein. The fusion GST-MYB305 protein was purified using Glutathione
Preparation of labeled oligonucleotide probes and mobility shift assays

The probes for analysis of the MYB binding sites in the \textit{nec1} promoter were prepared by $[^{32}\text{P}]$-labeling. The duplex N1S1 probe was prepared by annealing two single stranded oligonucleotides, N1P1-UPPER and N1P1-LOWER (Appendix A). The two oligonucleotides were mixed in a 0.2ml tube and heated to 95$^\circ$C using thermal cycler. Then, the tube was floated on a 250ml beaker filled with 95$^\circ$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 N1P2-LOWER. Both probes were labeled with gamma-$[^{32}\text{P}]$-ATP by T4 polynucleotide kinase (www.promega.com). 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 instruction (www.promega.com). The N5P probe for the analysis of the MYB binding site in the \textit{nec5} promoter was prepared using a fluorescent HEX tag incorporated during synthesis at the 5’ end of the oligonucleotide by IDT (www.idtdna.com). 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 (www.gelifesciences.com) instead of X-ray film.

2.2.8 \textit{nec1} promoter assays

Constructs

All clones were based on the \textit{nec1} promoter in the previously described vector, pRT454 (AF132671), (Carter et al., 1999; Thornburg et al., 2003). The promoter deletions were prepared by PCR using a method in which specific restriction endonuclease sites replaced the MYB S1 and S0 sites using six specific oligonucleotides. We prepared oligonucleotides p454-1 and p454-6 that were
complementary to sequences in the far upstream (-1216) and far downstream (+50) sequences of the *nec1* promoter, respectively. p454-1 had an *Apa*I site at its 5' end and p454-6 had a *Sac*I site at its 5' end. Oligonucleotide p454-3 was complementary to sequences immediately upstream of the S1 MYB site (-898) of the *nec1* promoter. p454-4 was complementary to sequences immediately downstream of the S1 MYB site (-892) of the *nec1* promoter. Both p454-3 and p454-4 had *Sac*II sites at their 5' ends. Likewise, p454-4 and p454-5 were complementary to sequences upstream and downstream of the S0 MYB site of the *nec1* promoter, respectively. Both p454-4 and p454-5 had *Spe*I sites at their 5' ends. All of these restriction sites, *Apa*I, *Sac*II, *Spe*I and *Sac*I are unique and are not found in the *nec1* promoter. All PCR reactions used pRT454 as a template.

For the *nec1* promoter lacking the S1 site, p454-1 and p454-2 were used to generate a 330-nucleotide fragment, with *Apa*I and *Sac*II ends, that corresponded to the far upstream portion of the *nec1* promoter. A second PCR reaction using p454-3 and p454-6 generated a 940-nucleotide fragment, with *Sac*II and *Sac*I ends, corresponding to the *nec1* promoter downstream of the S1 site. These fragments were gel purified and digested with the appropriate restriction sites (*Apa*I/*Sac*II or *Sac*II/*Sac*I) and cloned in a three-way ligation into *Apa*I/*Sac*I digested pGEM-T (www.promega.com) to generate pRT609. For the *nec1* promoter lacking the S0 site, p454-1 and p454-4 generated a 1.1 kB fragment corresponding to the *nec1* promoter upstream of the S0 site and p454-5 and p454-6 generated a 130 nucleotide fragment corresponding to the *nec1* promoter downstream of the S0 site. After gel purification, these fragments were digested with *Apa*I/*Spe*I or *Spe*I/*Sac*I and were cloned into *Apa*I/*Sac*I digested pGEM-T to generate pRT610. For the *nec1* promoter lacking both S1 and S0 sites, three PCR fragments were prepared that contained the far upstream portion of the promoter (p454-1 and p454-2), the middle portion (p454-3 and p454-4), and the far downstream portion (p454-5 and p454-6). After gel purification, these fragments were digested with *Apa*I/*Sac*II, *Sac*II/*Spe*I or *Spe*I/*Sac*I and were cloned into *Apa*I/*Sac*I digested pGEM-T to generate pRT611. These promoters (~1.25 kb in
size) were then moved into the pDONR/Zeo gateway entry vector (www.invitrogen.com) following PCR of the intermediate vectors, pRT609, pRT610, and pRT611 using the \textit{attB} containing oligonucleotides, p454-7 and p454-8 and BP clonase. The native \textit{nec1} promoter was also moved into the pDONR/ZEO entry vector using p454-7 and p454-8. The resulting entry vectors were sequenced from each end and found to be correct: the complete native \textit{nec1} promoter, pRT616; the \textit{nec1} promoter lacking the S1 site but retaining the S0 site, pRT617; the \textit{nec1} promoter retaining the S1 site but lacking the S0 site, pRT618; and the \textit{nec1} promoter lacking both S1 and S0 sites, pRT619. These promoters were subsequently moved into the gateway destination vector, pHGWFS7 (Karimi et al., 2002), using LR clonase to generate the promoter-GUS fusions shown in Figure 2.7 (inset). The destination vectors were again sequenced to ensure they had the desired structure.

\textbf{Plant transformation}

\textit{Nicotiana tabacum} cv. Xanthi plants were transformed with \textit{Agrobacterium tumefaciens} LBA 4404 (Ooms and Bakker, 1982), by co-cultivation as described (Thornburg et al., 1987). Transgenic progeny lines were selected on MS plates containing 0.1ug/ml naphthalene acetic acid, 1.0ug/ml benzylaminopurine, 50ug/ml Hygromycin B and 100ug/ml Carbenicillin. After regeneration, plantlets were transferred to MS media with 50ug/ml Hygromycin B and 100ug/ml Carbenicillin, but containing no hormones for rooting. Once the rooted shoots were about 5 cm tall, they were transferred into soil and grown to floral maturity in the greenhouse.

\textbf{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 \(\beta\)-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 (Bradford, 1976). The activities of GUS were determined by
fluorometric assays using 4-methylumbeliferyl-β-D-glucuronide (MUG) as substrate (Jefferson and et al., 1987).

2.2.9 LxS-MYB305 overexpression and LxS-MYB305 RNAi plant

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 (www.invitrogen.com) vector to obtain the entry clone, pRT613. This plasmid was subsequently recombined (LR reaction) into pMDC32 (Curtis and Grossniklaus, 2003) vector to form an over-expression plant transformation vector, pRT621. Transgenic plants were prepared as described above.

The 3’ end of the cDNA sequence of LxS-myb305 gene 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, pRT671. 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 ug/ml ammonium glufosinate instead of Hygromycin B in both selection and rooting stages.

2.2.10 RT-PCR and quantitative RT-PCR

RT-PCR

The tobacco nectaries were collected and frozen in liquid nitrogen; RNAs were then purified using the TRIZol Reagent method (www.invitrogen.com) according to the manufacturer’s instructions. The first strand cDNAs were made using the Eppendorf cMaster RT PCR System. All the primers used in this study are given in Appendix A. 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 over-expression Nicotiana tabacum cv. Xanthi and nectaries of the LxS-MYB305 RNAi Xanthi plants were collected and frozen in liquid nitrogen. Total RNA was purified using the TRizol Reagent method (www.invitrogen.com) 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 Appendix A. The PCR reactions were performed on Stratagene® mx4000 multiplex quantitative PCR system using the Brilliant® II SYBR® Green QPCR Master Mix (Stratagene). 18S and 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.

2.2.11 Anti-LxS-MYB305 and anti-NEC1 Antiserums

Rabbit anti-NEC1 antiserum was previously described (Carter et al., 1999). Rabbit anti-LxS-MYB305 antiserum was produced by GenScript (genscript.com) 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, catalog# 17-0981-01; gelifesciences.com). Twenty milliliters of crude antiserum were passed through the column and after washes with 100ml 50mN Tris-HCl pH 7.0, 100ml 10mN Tris-HCl pH 7.0 containing 0.5M NaCl, and finally with 100 ml 50 mM Tris-HCl, pH 7.0 with 0.2M NaCl, the anti-LxS-MYB305 antibodies were eluted with 100mM glycine pH 2.5. The antibodies were neutralized with 1M Tris-HCl pH8, 50% glycerol, 0.25M NaCl, 0.1M KCl prior to storage at -20°C and use in the immunoblot experiments.
2.2.12 Immunoblotting

Various tobacco tissues were collected and immediately frozen in liquid nitrogen. Proteins were isolated using a plant total protein extraction kit (#PE0230 www.sigmaaldrich.com) and 100 ug of total protein from leaves or different nectary stages were electrophoresed on a 12% SDS PAGE. After running the gel, the proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. The proteins were detected with either 1:2000 rabbit anti-LxS-MYB305 antiserum or 1:5000 anti-NEC1 antiserum, and then with HRP-conjugated goat anti-rabbit antiserum. The SuperSignal West Pico Chemiluminescent Substrate (Pierce Prod #34080 www.piercenet.com) was used to detect the signals on membranes; the membranes were then exposed to X-ray films. Short exposure times (15 to 30 minutes) ensured that analyses were conducted in the logarithmic phase of amplification. Individual exposure times are indicated in each figure legend. All exposures were complete within 1 hour after application of substrate to the blot unless otherwise indicated.

2.3 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, 2003b), we characterized this factor in detail.

2.3.1 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 2.1, this myb gene is strongly expressed only in flowers. It was not expressed in stems (lane 6) or 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
Figure 2.1. Temporal and spatial expression of *myb305* in *LxS8* plants.

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.
Based upon 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.

2.3.2 Phylogenetic Analysis

BLAST analysis (Altschul SF, 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 Antirrhinum majus MYB transcription factors MYB305 and MYB340 (Jackson et al., 1991), the Pisum sativum MYB26 (Uimari and Strommer, 1997), and the Gerbera hybrida MYB8 (Laitinen et al., 2005). Because the 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 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 2.2A, the MYB DNA-binding domains identify a distinct clade of proteins containing LxS-MYB305, and other closely related proteins including one each from Gerbera hybrida, and Pisum sativa, and two each from Antirrhinum majus (Am-MYB305 and Am-MYB340) and Arabidopsis thaliana 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.
Figure 2.2. Phylogenetic analysis of LxS-MYB305 protein.

Panel A. Analysis of the LxS-MYB305 protein DNA binding domain and the MYB DNA binding domains from *Antirrhinum majus*, *Arabidopsis thaliana*, *Gerbera hybrid cv. Terra Regina* and *Pisum sativum* MYBs. The alignment used for this analysis and GenBank accessions are provided in Appendix B. The shaded area shows a distinct clade of proteins containing LxS-MYB305 and its closely related proteins.

Panel B. Analysis of the complete protein sequences of LxS-MYB305 and its closely related MYBs. The alignment used for this analysis and GenBank accessions are provided in Appendix C.
To get an enhanced picture of this clade, the full protein sequences (DNA-binding domain plus activation domain) of the eight most closely related proteins were phylogenetically analyzed and are displayed in Figure 2.2B. As can be seen, LxS-MYB305 more closely resembles Gerbera hybrida MYB8 than the Antirrhinum majus MYBs, or Pisum 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.

2.3.3 Nuclear Localization of MYB305 protein

To evaluate the subcellular localization of MYB305 we utilized a transient transformation assay using tobacco mesophyll protoplasts. For these studies, the GFP reporter gene was fused in frame to the 5' end of the LxS-myb305 gene open reading frame (Figure 2.3A). The plasmid pMDC43 encoding GFP alone served as a control. Protoplasts transformed with pMDC43 showed GFP fluorescence throughout the entire cell (Figure 2.3B). In contrast, cells transformed with the full-length GFP:myb305 construct showed bright fluorescence localized to the nucleus (Figure 2.3C). To investigate which part of the MYB protein possessed the nuclear localization signal, LxS-myb305 cDNA was truncated into two parts, myb305Δ1 and myb305Δ2 (Figure 2.3A). myb305Δ1 encodes the R2R3 MYB DNA binding domain but not the transcriptional activation domain, whereas myb305Δ2 encodes the transcriptional activation domain but not the R2R3 DNA binding domain. The two truncated myb305 cDNA fragments were each fused in frame with GFP gene. The GFP:MYB305Δ1 fusion protein localized to the nucleus, whereas the GFP:MYB305Δ2 fusion protein did not (Figure 2.3D and E), indicating that the nuclear localization signal resides within the R2R3 domain.
Figure 2.3. Nuclear localization of LxS-MYB305 protein.

Panel A. The plasmid constructs used for the nuclear localization studies. The amino acid number of the included LxS-MYB305 fragments are shown above the hatched bars. The locations of the R2R3 DNA binding domains and the activation domain (AD) of the LxS-MYB305 protein are shown below the figure.

Panels B - E. Transient expression assay of GFP fluorescence in transformed N. tabacum cv. Xanthi mesophyll protoplasts. Typical results of tobacco protoplasts transformed with constructs encoding GFP only (pMDC43; Panel B), full length GFP:MYB305 fusion (pRT656; Panel C), truncated GFP:MYB305Δ1 fusion (pRT657; Panel D) and truncated GFP:MYB305Δ2 fusion (pRT658; Panel E).
2.3.4 MYB305 is a transcription activator in yeast

To investigate whether MYB305 can serve as a transcriptional activator, we utilized 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 2.4A). 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 a his3 reporter systems (Figure 2.4A) 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 2.4B, little growth was observed when the pDBleu strain (negative control) was grown on medium lacking histidine. In 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 encodes the GAL4 DNA binding domain fused with the complete LxS-MYB305 protein also resulted in strong growth of transgenic yeast. It also gave about 20-fold more β-galactosidase activity than the negative control in the quantitative lacZ reporter assay (Figure 2.4C). Based upon these data, we conclude that the LxS-MYB305 is a transcription activator and is capable of activating transcription in yeast.

2.3.5 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 2.4A, contain the entire R2R3 domain but no activation domain (amino acids 1 to 125; pRT631), the complete
Figure 2.4. LxS-MYB305 is a transcriptional activator in yeast and it activates the transcription of reporter genes via its C-terminal domain.

Panel A. Constructs used in these studies. The GAL4 DNA binding domain (DB) and LxS-MYB305-Δ fragment (MYB-Δ) fusion genes driven by the ADH1 promoter serve as effectors; the HIS3 and LacZ (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; the AD represents the activation domain. The +control contains a recombined Gal4 DNA binding domain and activation domain; the –control contains Gal4 DNA binding only.

Panel B. Serial-dilution growth assays of transformed yeast on selective YSM His− plus 10 mM 3AT medium. Constructs are identified to the left of the figure. Dilutions are identified below the figure.

Panel C. Quantitation of β-galactosidase activity in yeast. Constructs are identified below the bars. Means + SD (n=4 with 2 replicates for each sample). +: positive control construct; −: negative control construct.
A

**Reporter**

UAS (gal4)  |  HIS3

**Effector**

ADH1 Promoter  |  GAL4DB  |  MYB+Δ  |  ADH1 Terminator

- pRT624
- pRT631
- pRT632
- pRT633
- pRT634
- pRT635

(-His +10mM 3AT)

B

(+control)  |  (-control)

pRT624  |  pRT631  |  pRT632  |  pRT633  |  pRT634  |  pRT635

Dilutions

C

β-gal Activity (units)

+  |  -

pRT624  |  pRT631  |  pRT632  |  pRT633  |  pRT634  |  pRT635
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), the C-terminal end of the activation domain (amino acids 169 to 192; pRT634), and the entire LxS-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 2.4B, yeast cells harboring pRT631, which lacks the complete activation domain, do not grow well on media lacking histidine. In 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 LxS-MYB305 protein 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 2.4C) 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.

2.3.6 Acidic residues near the MYB305 C-terminus mediate transcriptional activation

To further evaluate the function of the activation domain, we analyzed its predicted secondary structure (amino acids 117 to 192) using Jpred, a neural network-based program, (James and Geoffrey, 1999, 2000) that identifies structural homologs, performs multiple sequence alignment, and classifies each residue as likely to reside in an alpha helix, beta sheet or random coil secondary structure. As shown in Figure 2.5A, 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 alpha helix that is 7 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 alpha 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 2.5B), the hydrophobic residues Met$^{180}$, Ile$^{183}$, Trp$^{184}$, and Met$^{186}$ lie on one face of the alpha 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 alpha helix. Because amphipathic alpha 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 post-translational 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
Figure 2.5. Analysis of the MYB305 activation domain.

Panel A. Alignment of amino acid sequences of proteins closely related to LxS-MYB305. The amino acid numbers in LxS-MYB305 are indicated above the sequences and the bar represents the minimal activation domain identified in Figure 2.4. Regions of identity are shaded. The secondary structural predictor “Jpred” predicts only one region of secondary structure, a single alpha helix located in the center of the minimal activation domain.

Panel B. Surface analysis of the predicted alpha helix showing the hydrophobic and hydrophilic surfaces. The positions of Met$^{180}$ and Met$^{186}$ are identified.

Panel C. Site-specific mutagenesis of the minimal activation domain of the LxS-MYB305. Serial-dilution growth assays of transformed yeast on selective YSM Ur$^{-}$ medium. Effector and reporter constructs are identical to those in Figure 2.4. Positive (Gal4DB-AD) and negative (pDBleu) controls are shown. The site-specific mutants are identified to the left of the figure with construct numbers shown at right. The location of the predicted alpha helix is shaded and shown below these sequences. The spots are serial dilutions of each transformed yeast strain.
Ser\textsuperscript{185} were changed to Ala. In construct pRT653, the acidic residues Glu\textsuperscript{181}, Asp\textsuperscript{182}, and Asp\textsuperscript{192} were changed to Ala. In pRT654, all five residues were changed to alanine. As shown in Figure 2.5C, changing the serine residues to alanine in construct pRT648 had no effect on the transcriptional activation of the modified protein, suggesting that phosphorylation of these serine residues may not be important for transcriptional activation. In contrast, changing the acidic residues to alanine 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.

2.3.7 MYB305 binds the nec1 and nec5 promoters in vitro

Nectarin 1 (NEC1) is the major protein secreted into nectar of ornamental tobacco (Carter et al., 1999; Carter and Thornburg, 2000a). 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, 2003a). 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 the current study, we re-examined the nec1 promoter sequence and identified a second potential MYB binding site located at -86 to -78 of the nec1 promoter (Figure 2.6B). To evaluate whether LxS-MYB305 protein can bind to these regions of the nec1 promoter, we expressed this transcription factor as a GST protein fusion in E. 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 2.6A, 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 as no reactivity against any proteins present in the foliage of the ornamental tobacco plants (Lane 7).
Figure 2.6. MYB305 binds to the nec1 and nec5 promoter fragments.

Panel 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. Lane 4 to Lane 7 are immunoblots using purified anti-LxS-MYB305 antibodies: Lane 4, purified GST-MYB305 protein; Lane 5, GST protein; Lane 6, 100ug of protein from Stage 12 nectary tissue of wildtype LxS8 plant; Lane 7, 100ug of protein from foliage of wildtype LxS8 ornamental tobacco plant.

Panel B. Structure of the Nicotiana plumbaginifolia nec1 promoter showing the location and sequences of the two consensus MYB binding sites, S1 and S0.

Panel 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 Appendix A.

Panel 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 Appendix A.

Panel E. Structure of the Nicotiana plumbaginifolia nec5 promoter showing the location of the consensus MYB binding site.

Panel 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 Appendix A.
This purified GST-MYB305 fusion protein was then used in electrophoretic mobility shift assays to evaluate its interactions with [32P]-labeled DNA probes. Two duplex oligonucleotide probes were utilized for these analyses, the S1 probe (N1S1; Appendix A) 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 (Figure 2.6C 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 9 nucleotide MYB consensus sequence was replaced with a poly A/T track (Appendix A). As shown in Figure 2.6, the addition of these unlabeled competitor probes had no effect on the binding of the GST-MYB305 to the original labeled probes. Based upon 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 (see Figure 2.6E) (Carter and Thornburg, 2003a). Therefore, we tested the purified GST-MYB305 for interaction with a synthetic nec5 promoter element. As shown in Figure 2.6F, GST-MYB305 did indeed interact specifically with the nec5 MYB binding site. This binding could be competed with an excess of unlabeled N5P probe, but not with the unlabeled 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 Figure 2.6, Panels C and D 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 utilized 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 (Appendix D) 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.

2.3.8 Wildtype, but not mutant, nec1 promoters drive GUS expression

To evaluate the role of the MYB binding sites in regulating transcription from the nec1 promoter, we generated a series of promoter-GUS constructs that contained the wildtype nec1 promoter, or the nec1 promoter with a mutation at the S1 site, at the S0 site or at both sites (Figure 2.7, 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 2.7, four transgenic plants, each harboring the wildtype nec1 promoter-GUS fusion construct, all expressed between 2300 and 4400 units of GUS activity per mg nectary protein. In 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
Figure 2.7. Analysis of wildtype and mutant nec1 promoters in transgenic Nicotiana tabacum cv Xanthi plants.

Different promoter constructs were transformed into Xanthi plants and mature floral nectaries of transgenic plants were assayed for β-glucuronidase activity. Inset shows the clone number and structure of the various constructs tested. The MYB site mutations were effected by deletions of the binding site: pRT627 contains the full length unmodified promoter (-1216..+50); pRT628 contains the full nec1 promoter with S1 MYB site deleted (-1216..-898 + -892..+50); pRT629 contains the full nec1 promoter with the S0 MYB site deleted (-1216..-84 + -78..+50); pRT630 contains the full nec1 promoter with both MYB sites deleted (-1216..-898 + -892..-84 + -78..+50). GUS activity is presented in units/mg of nectary protein for four independent transformants for each of the four constructs. Means + SD are shown (n = 6).
Figure 2.8. Temporal expression of MYB305 in nectaries of wildtype LxS8 ornamental tobacco plants.

Panel A. RT-PCR of myb305, nec1 and nec5 genes in the nectary glands of LxS8 plants at different floral stages. Both 18S and 26S rRNAs were utilized as controls. PF = 48 hours post-fertilization.

Panel B. Protein blotting of MYB305 and NEC1 proteins in the nectary glands of LxS8 plants at different floral stages.
with our earlier observation that deletion of the upstream site results in a dramatic loss of promoter activity (Carter and Thornburg, 2003b).

2.3.9 Temporal expression of myb305, nec1, and nec5

We have provided evidence that MYB305 protein may function as a transcriptional regulator of nec1 expression. The MYB305 protein is nuclear localized, it activates transcription in yeast, it binds to nec1 and nec5 promoter sequences in vitro, and mutations of the binding sites in the nec1 promoter destroy promoter activity in vivo. If MYB305 does indeed regulate nec1 and nec5 expression in plants, it should be expressed prior to either of these genes. Therefore, we used RT-PCR to evaluate when the nectarin genes and myb305 gene are expressed during nectary development.

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 gene 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 utilized 18S and 26S rRNAs as loading and PCR controls. As shown in Figure 2.8A, 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 2.1. After fertilization, myb305 is abruptly down regulated. In 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, 2003b, 2004b). Figure 2.8B shows MYB305 and NEC1 protein levels by immunoblotting. These protein blots confirm the expressions of myb305 and nec1 protein in Figure 2.8A. Together, these results demonstrate that myb305 gene 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.

2.3.10 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 cv Xanthi 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, phenylalanine ammonia lyase (pal) and chalcone isomerase (chi) have previously been shown to be regulated by the Antirrhinum majus MYB305 (Sablowski et al., 1994; Sablowski et al., 1995), so we also evaluated expression of these genes in the foliage of these plants.

For this study, we developed four independent transgenic over-expression (OX) plant lines that express the coding region of LxS-myb305, under the control of the strong CaMV 35S promoter. The phenotype of the OX plants was similar to the wildtype plants, with the exception that they were shorter, growing to approximately 50% the height of wildtype plants (Appendix E). As shown in Figure 2.9A quantitative RT-PCR analysis demonstrates that none of the genes of interest were highly expressed in the foliage of wildtype, untransformed tobacco plants. These results confirm the expression pattern of myb305 shown in Figure 2.1 as well as the published patterns of expression previously observed for nec1 and nec5 (Carter et al., 1999; Carter and Thornburg, 2003b, 2004b). As opposed to the wildtype plants, the myb305 mRNA is detectable in the foliage of each of the over-expression lines. Similarly, nec1 and nec5 transcripts were found in the over-expression lines. Except one line, OX1, which showed no apparent positive signal for nec5, all other lines showed substantial levels of nec1 and nec5 expression in the MYB305 over-expression lines. In addition, we also tested the expression of the flavonoid genes, pal and chi. Neither of these flavonoid genes was expressed
Figure 2.9. Analysis of LxS-MYB305 over-expression and RNAi plants.

Panel A. Quantitative RT-PCR analysis of LxS-myb305, nec1, nec5, pal and chi expression in the foliage of LxS-MYB305 over-expression Xanthi plants. Means + SD are shown.

Panel B. Quantitative RT-PCR analysis of LxS-myb305, nec1, nec5, pal and chi expression in the stage 12 nectary glands of LxS-MYB305 RNAi Xanthi plants. Means + SD are shown.

Panel C. Immunoblotting of MYB305 and NEC1 proteins in the foliage of LxS-MYB305 over-expression Xanthi plants.

Panel D. Immunoblotting of MYB305 and NEC1 proteins in the stage 12 nectary glands of LxS-MYB305 RNAi Xanthi plants.

Panel E. Phenotype comparison of wildtype and MYB305 RNAi plant nectary. Scalebar = 2mm.
in the foliage of wildtype tobacco plants (Figure 2.9A), but both were observed in each of the MYB305 over-expression 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 to nec1 and nec5, neither Nectarin III (nec3) nor Nectarin IV (nec4) was expressed in the foliage of these plants (Appendix F) suggesting that these genes are not regulated by MYB305. These results for MYB305 and NEC1 were confirmed at the protein level by immunoblotting analysis. Wildtype plants showed undetectable levels of both MYB305 and NEC1 (Figure 2.9C). Again, these results are internally consistent with our earlier observations (Figure 2.7A) and with previously published observations (Carter et al., 1999; Carter et al., 2007a). 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 wildtype plants failed to show any bands, even with these long exposures.

2.3.11 RNAi knockdowns of MYB305 also knocks down expression of nec1, and nec5

Finally, we also prepared a series of RNAi lines that showed reduced expression of myb305 in the nectary. qRT-PCR analysis of Stage 12 nectaries from wildtype plants (Figure 2.9B) show strong expression of myb305, both nec1 and nec5, as well as both of the flavonoid biosynthetic genes (pal and chi) in the wildtype plants. Again, these data are consistent internally with our earlier observations (Figures 2.1 and 2.8) as well as with previously published observations (Moyano et al., 1996; Carter et al., 1999; Carter and Thornburg, 2003b, 2004b). 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 2.9D). While the wildtype 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 nectaries of these RNAi plants reflect the levels of nectary-expressed myb305 and nec1 mRNAs.

2.4 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, 2003b). In the current study, we have 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 protein is closely related to snapdragon MYB305 and homologues from Gerbera hybrida and Pisum sativa, as well as to a group of MYB proteins from Arabidopsis thaliana (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 phenylalanine ammonia lyase (pal), chalcone isomerase (chi), and 4-coumaryl-CoA-ligase (4cl) 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 2.2). These two proteins, MYB305 and MYB340, appear to be regulated by phosphorylation in Antirrhinum flowers (Moyano et al., 1996).
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 (Li et al., 2006; Mandaokar et al., 2006). In Arabidopsis they are required for proper anther development (Mandaokar et al., 2006). It has also been shown that over-expression 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 (Lu 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 wildtype, 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 (Appendix G). Both the OX and the RNAi lines had shorter anthers than the wildtype, with the RNAi lines being more severely affected. Likewise, the style length for each of the mutant lines was also shorter than the wildtype; 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 analysed the ornamental tobacco sequence together with only the rosid sequences (from pea and *Arabidopsis*). As shown in Appendix D, 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 AR,
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 protein 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 1-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, the current 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 alpha helix located near the middle of the 23 amino acid activation region. This alpha helix was found to be amphipathic with hydrophilic residues exposed on one side of the helix and hydrophobic residues on the other. Such alpha helices are often form 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 serine 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 alanine substitutions of two serine residues did not alter transcriptional activation; however, replacement of three acidic residues with alanine 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 wildtype or mutagenized nec1 promoters. In transgenic plants we found that the wildtype 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...
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 levels 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 the *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; Uimari 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 (Appendix F). 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, 2004d) (Naqvi et al., 2005b) and these studies confirm this.

Finally, we have 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 beta-carotene as wildtype nectaries do. These plants also show reduced levels of nectar accumulation in the flower. Both of these characteristics (beta-carotene accumulation and nectar production) are associated with starch metabolism in nectaries (Horner et al., 2007; Ren et al., 2007a; Ren et al., 2007d). 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 \textit{in vitro}. Furthermore, mutation of the MYB binding sites within the nec1 promoter destroys promoter activity \textit{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. Further, 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.
Chapter 3. Knockdown of MYB305 inhibits the nectary maturation and petal expansion by disrupting starch metabolism

A paper to be submitted to *The Plant Journal*

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**Abstract**

MYB transcription factors play important roles during the floral organ development. In this study, we generated MYB305 RNAi knockdown ornamental tobacco plants and studied the roles of MYB305 in the growth of floral organs. The MYB305 RNAi plants produced flowers with abnormal nectary and petal development. In the MYB305 RNAi plants, the nectaries show juvenile character at late stages and secrete reduced levels of nectar, the petals are smaller and do not fully expanded during anthesis. Because normal nectary development is associated with starch metabolism, we examined starch accumulation in the floral organs. This study showed that in the MYB305 RNAi plants, the starch accumulation was reduced in both the nectary, before nectar secretion, and in petals, before anthesis; and the starch degradation was disrupted in the nectaries during nectar secretion. Furthermore, the starch metabolic genes expression patterns are altered in the RNAi nectaries at late flower stages. These results suggest that MYB305 plays important roles during the morphogenesis of petals and during the maturation of nectaries in ornamental tobacco plants by controlling the expression of starch metabolic genes, through the synthesis of starch, which is the main carbohydrates source for petal expansion and nectary maturation.

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3.1 Introduction

Starch is crucial for normal plant growth and development. It is the major and the most abundant storage carbohydrate in plants (Orzechowski, 2008). Starch is synthesized inside plastids and is usually deposited in the form of insoluble granules (Zeeman et al., 2002; Smith et al., 2005). In the photosynthetic tissues, transient starch granules are synthesized in the chloroplasts during the day by capturing glucose made through photosynthesis and are degraded at night to provide sugars for other biological processes (Zeeman et al., 2002; Smith et al., 2005). In some nonphotosynthetic tissues, termed as sink organs, starch is synthesized in a form of storage starch in the amyloplasts (Ren, 2007). This storage starch accumulates to high levels and forms a reserve source of metabolites and energy for later use (Bieleski, 1995; Fernie et al., 2002). The tobacco nectary gland is such a sink tissue that accumulates large amount of starch during the flower development.

The tobacco nectary is a specialized organ that attracts visiting pollinators which transfer gamete from flower to flower by rewarding them with a carbohydrate rich nectar (Carter et al., 1999). The nectary grows larger as flower develops and the increasing size of the nectary gland is accompanied by the accumulation of starch granules in nectary amyloplasts (Ren et al., 2007c). Compared to the low levels of starch at early stages, high levels of starch is accumulated in the nectary parenchyma cells at late stages and reaches a peak approximate 24 hours before anthesis. After this point, the nectary starch is rapidly degraded to provide soluble carbohydrates for nectar secretion and substrates for antioxidant production (Horner et al., 2007; Ren et al., 2007c). Along with starch accumulation and degradation processes, the expression pattern of starch metabolic genes is also changed. The starch anabolic genes, including ADP-glucose pyrophosphorylase (AGPs) and Starch synthase 3 (SS3), are strongly expressed in the starch accumulation stages but not in the degradation stages. In contrast, the starch catabolic genes, including isoamylase1 (ISA1), α-amylase (AAM) and β-amylase
(BAM) are not expressed in starch accumulation stages, but are strongly expressed in starch degradation stages (Ren et al., 2007b).

To study the role of MYB305 in the starch metabolism, we developed MYB305 RNAi knockdown ornamental tobacco plants that showed reduced levels of MYB305 and we studied the resulting plants in several aspects. First, we studied the phenotypic changes. In the flowers of the RNAi plants, the nectary shows juvenile characters. In addition, we observed that the petals are not able to fully expand. Second, we analyzed carbohydrates and starch content in both nectaries and petals. The results show that starch metabolism is disrupted in both nectary and petals of MYB305 RNAi plants. Third, we showed that altered starch metabolism in floral tissues correlates with starch metabolic gene expression in MYB305 RNAi plants. Our results demonstrate that MYB305 knockdown inhibits nectary maturation and petal expansion by disrupting starch metabolism. Thus, MYB305 plays an essential role in normal flower development.

3.2 Materials and Methods

3.2.1 Materials

Unless otherwise noted, the chemicals used in these studies were obtained from either Fisher Chemical Co (www.fishersci.com) or Sigma Chemical Co (www.sigmaaldrich.com) and were of the highest quality available.

3.2.2 Plants

For the wildtype LxS8 ornamental tobacco plants used in these studies, the growth condition 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 (Koltunow et al., 1990).

The MYB305 RNAi LxS8 ornamental tobacco plants were generated by cloning LxS-myb305 cDNA sequence into pHellsgate2 RNAi vector (Wesley et al.,
2001; Helliwell and Waterhouse, 2003) followed by subsequent plant transformation. The \textit{LxS-myb305} cDNA sequence (AF132671) was PCR amplified by attBMYB-1 and attBMYB-2 primers and then recombined (BP reaction) into the pHellsgate2 vector to obtain transformation vector, pH2M. This plasmid serves as transformation vector and were transformed into \textit{N. langsdorffii X N. sanderae (LxS8)} ornamental tobacco plants with \textit{Agrobacterium tumefaciens} LBA 4404 (Ooms and Bakker, 1982), by co-cultivation as described (Thornburg et al., 1987). Transgenic progeny lines were selected on MS plates containing 0.1ug/ml naphthalene acetic acid, 1.0ug/ml benzylaminopurine, 50ug/ml Kanamycin and 100ug/ml Carbenicillin. After regeneration, plantlets were transferred to MS media with 50ug/ml Hygromycin B and 100ug/ml Carbenicillin, but containing no hormones for rooting. Once the rooted shoots were about 5 cm tall, they were transferred into soil and grown to floral maturity in the greenhouse.

3.2.3 Genes and accession numbers

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 \textit{LxS-MYB305} cDNAs was previously described (Carter and Thornburg, 2004d) (Naqvi et al., 2005b). The sequence of the \textit{LxS8-MYB305} cDNAs were deposited in GenBank with accession numbers EU111678 and EU111679. Other genes used in this study include \textit{LxS8 ADP-glucose pyrophosphorylase small subunit (AGPs, DQ021458)}, Starch Synthase 1 (SS1, DQ021463), Starch Synthase 3 (SS3, DQ021464), alpha-Amylase (AAM, DQ021455), beta-Amylase (BAM, DQ021457), Isoamylase 1 (ISA1, DQ021461) and Isoamylase 3 (ISA3, DQ021471).

3.2.4 Carbohydrate analysis

The nectar was collected from flowers as previously described (Carter et al., 1999; Ren et al., 2007c). To insure all of the nectar is collected, 200ul of water was used to wash the floral tubes, nectaries, and receptacles. This wash was combined with the isolated nectar. The samples were used immediately for carbohydrate
quantification. The levels of sucrose, glucose, and fructose were evaluated using The Sucrose/D-Glucose/D-Fructose Determination Kit (Boehringer Mannheim/r-Biopharm, Cat# 10716260035), according to the manufacturer's directions. Each flower represents a single sample and 10 independent samples were measured for each wildtype or transgenic plant.

To quantify free soluble carbohydrates in nectaries, the nectaries were separated from the gynoecia, then 20 nectaries from the same plant were pooled together to represent a single sample and 4 independent samples were measured for each wild-type or transgenic plant. Each sample was homogenized by grinding in a ConTorque tissue homogenizer (Eberbach Corp, Ann Arbor, MI) for 5 min, the homogenate was then brought to a final volume of 10 mL with water to ensure complete dissolve of soluble carbohydrates. Following centrifugation (15,000 x g) for 10 min, the clarified homogenates were used immediately for carbohydrate quantification. The levels of sucrose, glucose, and fructose were evaluated using The Sucrose/D-Glucose/D-Fructose Determination Kit (Boehringer Mannheim/ r-Biopharm, Cat# 10716260035), according to the manufacturer's directions.

3.2.5 Starch analysis

To isolate starch, a published method (Vasanthan, 2001) was used in this study. Twenty nectaries were separated from gynoecia and pooled together in each isolation, four independent isolations were measured for each wild-type or transgenic plant at flower Stage 9 and Stage 12 as previously described (Carter and Thornburg, 2000a). Each sample containing 20 nectaries were frozen in liquid nitrogen and homogenized in a mortar and pestle. To isolate starch, 5 mL of 80% ethanol was added to each nectary homogenate, then incubated for 5 min in 80 °C water bath, followed by centrifugation for 10 min at 1,000 x g, room temperature and the ethanol was gently decanted so as to not disturb the pellet. The remaining pellet was dissolved in 1 mL of 90% aqueous DMSO and heated for 5 min in a boiling water bath. Subsequently the solubilized samples were diluted into 10 ml with water for enzymatic quantitation. All samples were processed at the same
time. Starch was quantified using the Starch Determination Kit from Boehringer Mannheim/r-Biopharm (Cat# 10207748035) according to the manufacturer's instructions with one modification. To better digest the starch, additional 1 unit of amyloglucosidase per reaction (Sigma, Cat. No.10130) was incubated with the starch for 17 h at 65 °C (Zhang et al., 2005) prior to quantification of hydrolyzed glucose. The petal starch quantification was performed similarly, except that 5 flower petals were used in each sample and the fresh weight of each sample were measured before being frozen in liquid nitrogen.

3.2.6 β-carotene analysis

Extraction of beta-carotene

To isolate the beta-carotene from nectaries, a direct plant pigments extraction method was used. 20 nectaries from the same plant were pooled together to represent a single sample for extraction, 4 independent samples were studied for each wildtype or transgenic plant at flower stage 12 as previously described (Carter et al., 1999; Ren et al., 2007c). One ml of acetone and ~50mg of anhydrous sodium sulfate were added to the sample containing 20 nectaries. The sample was then thoroughly homogenized. Then the organic layer was removed, and the process was repeated, another 1 ml aliquot of acetone, then with 1 ml aliquot of hexane. The three organic layers were combined, dehydrated with sodium sulfate, and evaporated to dryness, taken up in 50μl hexane and used for analysis. The beta-carotene was measured at 452nm. Thin layer chromatography (TLC) was performed on silica gel plates using a 9:1 hexane:acetone solvent as previously described (Horner et al., 2007).

3.2.7 RT-PCR and quantitative RT-PCR

RT-PCR

The nectaries or petals from LxS8 plants and transgenic LxS8 plants were collected and frozen in liquid nitrogen; RNA were purified using the TRIzol Reagent (www.invitrogen.com) according to the manufacturer’s instructions. The first strand cDNAs were made using the Eppendorf cMaster RT PCR System. All the primers
used in this study are given in Appendix A. 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 nectaries of the MYB305 RNAi *LxS8* plants were collected and frozen in liquid nitrogen. Total RNA was purified using the TRizol Reagent method (www.invitrogen.com) 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 Appendix A. 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.

**3.2.8 Starch staining analysis**

To compare starch content in developing ornamental tobacco flowers, the starch was detected by a previously described iodine staining method (Zhang et al., 2005). The flowers at different stages were collected at the same time. Then the harvested flowers were boiled in 100 mL 80% ethanol for 10 min to decolorize. The decolorized flowers were subsequently stained with a freshly prepared solution of I₂/KI (10g KI and 1g I₂ in 1 Liter of water) for 5 min, destained in water for 15 min, and photographed immediately.
3.2.9 Sugar feeding test

Staged flowers were collected from greenhouse grown plants by cutting through the pedicel close with a razor blade, immediately taken to the laboratory and the staged flowers were maintained on ice. The cut end of each flower was placed into 200 ml of artificial phloem feeding solutions. The composition of artificial phloem feeding solutions is: F-rich solution (20% fructose, 0.5% glucose and 0.5% sucrose), G-rich solution (20% glucose, 0.5% fructose and 0.5% sucrose) and S-rich solution (20% sucrose, 0.5% glucose, and 0.5% fructose). The flowers were grown at room temperature with adequate light and were photographed at different time intervals.

3.3 Results

The development of ornamental tobacco flowers can be divided into 12 different stages (Koltunow et al., 1990). From Stage 1 to Stage 6 is prematuration stage where all floral organs formed and start to grow. From Stage 7 to Stage 9 is maturation stage where floral organs grow rapidly and most floral organs reach the normal size that matured floral organs should be at the end of maturation stage. From Stage 10 to Stage 12 is postmaturation stage where most floral organs are fully developed and become functional, flowers start opening and enter anthesis. In developing flowers of ornamental tobacco plants nectary, starch accumulates to very high levels, reaching a peak amount about 24 hours before anthesis (Ren, 2007; Ren et al., 2007a; Ren et al., 2007c). Thereafter, starch is degraded rapidly and is converted to sugars for nectar production (Ren et al., 2007c) and as substrates for antioxidants (Horner et al., 2007). In a recent characterization of RNAi plants with reduced expression of the nectary transcription factor MYB305, we noticed that the flowers of those plants did not produce nectar (Liu et al., 2009). To determine whether the failure to produce nectar in these RNAi plants is related to altered starch metabolism, we began this investigation.
3.3.1 MYB305 knockdown caused aberrant nectary development

MYB305 is expressed in ornamental tobacco flowers primarily in the nectary and at lower levels in the floral ovary, floral tube and petals. MYB305 begins to be expressed in the nectary after Stage 6 and continues to be expressed throughout the remainder of floral development. To evaluate the morphological phenotypes of the wildtype and the MYB305 RNAi plants, we examined the flowers and gynoecia of both Stage 6 flowers, just prior to MYB305 expression, and Stage 12 flowers, at anthesis. These floral tissues are shown in Figure 3.1.

In the Figure 3.1, panels A to C show the Stage 6 wildtype ornamental tobacco flower, nectary and longitudinal cut nectary. Similarly, panels D to F show RNAi line 1 and panels G to I show RNAi line 2. The overall shape, including length, color and shape, do not differ between wildtype and RNAi line flowers at Stage 6 (Figure 3.1, panel A, D and G). The whole gynoecia were also examined, all three gynoecia showed similar size, shape and color (Figure 3.1, panel B, E and H). The exteriors of nectaries show the light yellow coloration found in normal Stage 6 nectaries (Horner et al., 2007). Panel C, F and I of Figure 3.1 (the longitudinal cut gynoecia) show that no differences were observed in either the nectaries or ovaries at Stage 6 when MYB305 is just beginning to be expressed.

At Stage 12, the wildtype and RNAi flowers show noticeable differences. Phenotypes of Stage 12 wildtype ornamental flower, nectary and longitudinal cut nectary are shown in the Figure 3.1 panels J to L. Likewise, the RNAi line 1 and RNAi line 2 are shown in the panels M to O and panels P to R respectively. The RNAi plant flower petals are not able to fully expand at Stage 12 in either RNAi line. This petal expansion failure not only occurs at anthesis, but this failure also last until abscission (Figure 3.7, panel B). When we examine the flowers, we observed that the wildtype nectary are dark orange color, while the RNAi plant nectaries are light yellow color (Figure 3.1, panel K, N and Q), which is approximately the normal color of Stage 6 wildtype nectaries. The dissected gynoecia confirm the color differences between Stage 12 wildtype and RNAi nectaries are throughout the
Figure 3.1 Phenotypes of wildtype and MYB305 RNAi LxS8 ornamental tobacco plants flower and nectaries.

Panel A-I, Stage 6 flowers and nectaries. Panel A-C show wildtype flower bud, nectary and longitudinal cut gynoecia; Panel D-F show flower bud, nectary and longitudinal cut gynoecia of MYB305 RNAi line 1; Panel G-I show flower bud, nectary and longitudinal cut gynoecia of MYB305 RNAi line 2.

Panel J-R, Stage 12 flowers and nectaries. Panel J-L show wildtype flower bud, nectary and longitudinal cut gynoecia; Panel M-O show flower bud, nectary and longitudinal cut gynoecia of MYB305 RNAi line 1; Panel P-R show flower bud, nectary and longitudinal cut gynoecia of MYB305 RNAi line 2.
whole nectaries instead of on the surface of the nectary (Figure 3.1, panel L, O and R). In summary, the MYB305 knockdown did not cause phenotypic change at Stage 6, but result in the retention of several juvenile phenotypes in the of MYB305 knockdown plant flowers at Stage 12. These retained juvenile characters include nectary color, petal size and flower shape.

3.3.2 β-carotene synthesis is decreased in MYB305 knockdown nectaries

As flowers develop, the nectaries of ornamental tobacco plants become colored a rich pumpkin orange. The pigment responsible for the orange coloration is β-carotene (Horner et al., 2007). It has been proposed that the biosynthesis of β-carotene is governed by availability of substrate molecules arising from starch breakdown (Horner et al., 2007; Ren, 2007). The direct comparison of wildtype and RNAi line Stage 12 nectaries shows that the orange color in the wildtype is much more intense than in any of RNAi line nectaries. To quantify this observation, we extracted the β-carotene from Stage 12 nectaries and measured the relative absorbance value at its peak absorbance wavelength, 452nm. As seen in Figure 3.2 panel A, the RNAi line nectaries have significant lower level of β-carotene, especially in the RNAi line 3 which only produces about 10% β-carotene of the wildtype. To exclude the possibility that other carotenoids might absorb at 452nm, we ran the thin layer chromatography on these samples (Figure 3.2, panel B). The separated β-carotene bands on the TLC plates clearly indicate that the wildtype nectaries indeed have more β-carotene content than RNAi lines and quantification of band intensity show this increase range from 5 to 10 folds. Thus, we conclude that MYB305 knockdown result in decreased β-carotene production in tobacco nectaries at late stages.

3.3.3 Reduced nectar secretion and decreased carbohydrates production occur in the MYB305 knockdown plant nectaries

The primary function of the nectary is to secrete nectar to attract visiting pollinators that will transfer pollen from flower to flower. The juvenile phenotypes observed in the MYB305 RNAi flowers suggested that nectar composition and
Figure 3.2 Comparitive study of β-carotene.

Panel A. Relative β-carotene production by absorbance at 452nm
Panel B. Thin layer chromatography of β-carotene
Figure 3.3 Nectar production and carbohydrates analysis.

Panel A. Nectar production in wildtype and MYB305 RNAi ornamental tobacco plants. Mean + SD are shown in each panels

Panel B. Nectar carbohydrates quantification. The areas of each circle represent total carbohydrates measured in this study and each circle was divided into three segments to illustrate the proportions of fructose, glucose and sucrose.
quantity may be affected. Therefore, we examined floral nectar production and composition to provide additional information about nectary function. In the wildtype ornamental tobacco flowers, nectaries begin secreting nectar at about Stage 10, 24h prior to anthesis, and reach a maximum at anthesis (Stage 12). Therefore, we first measured the volumes of nectar at these stages in wildtype and each of the four MYB305 RNAi lines. As shown in the Figure 3.3, panel A, very little nectar was observed in any flowers at Stage 10, whether they were wildtype or RNAi lines. However, at Stage 12, the wildtype nectaries secrete significantly higher amounts of nectar than any RNAi line nectaries. These indicate the nectar secretions were affected in the MYB305 RNAi plant nectaries.

To understand the difference of nectar compositions between wildtype and RNAi plants, we quantified three richest carbohydrates, glucose, fructose and sucrose, in the tobacco nectar at Stage 12. In the Figure 3.3, panel B, the areas of each circle represent total nectar carbohydrates and each circle was divided into three segments to illustrate the relative proportions of glucose, fructose and sucrose. The results show clear difference of carbohydrate quantity between wildtype and RNAi lines, all the three carbohydrates amounts are much higher, as high as 5 times, in the wildtype than in any of the RNAi plants. However, the concentrations of these three carbohydrates in the RNAi plant nectar are similar to the wildtype and the carbohydrate ratio, glucose:fructose:sucrose = 1:1:1 (molar ratio), in the RNAi lines is almost the same as in the wildtype (Ren, 2007).

3.3.4 Nectaries of MYB305 knockdown plants have reduced carbohydrate levels and changed carbohydrate compositions

Since the secreted nectar show significantly lower sugars in the RNAi plants than in the wildtype, we looked for similar differences in the floral nectaries. We extracted the soluble sugars from Stage 9 and Stage 12 nectaries and quantified the major carbohydrates: glucose, fructose and sucrose. In the Figure 3.4, panel A, the areas of each circle represent total carbohydrates as well as the proportions of glucose, fructose and sucrose. At Stage 9, the soluble nectary sugars and each of
Figure 3.4 Nectary carbohydrates analysis.

Panel A. Glucose, fructose and sucrose are quantified in the wildtype and RNAi plant nectaries at Stage 9 (left) and Stage 12 (right). The total carbohydrates are displayed in the areas of each circle. Each circle was divided into three segments to illustrate the relative proportions of glucose, fructose and sucrose.

Panel B. Glucose:Fructose:Sucrose (G:F:S) molar ratio in the nectaries of wildtype and RNAi Plants at different stages.
B. Nectary carbohydrates molar ratio (glucose:fructose:sucrose).

<table>
<thead>
<tr>
<th>G : F : S</th>
<th>Stage 9</th>
<th>Stage 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
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<td>1 : 0.95 : 1.05</td>
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<tr>
<td>RNAi-1</td>
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<td>1 : 0.97 : 0.56</td>
</tr>
<tr>
<td>RNAi-4</td>
<td>1 : 0.99 : 0.84</td>
<td>1 : 1.04 : 0.42</td>
</tr>
</tbody>
</table>
the three major nectary sugars were found to accumulate to higher levels in the wildtype than in the RNAi lines. At Stage 12, the difference of total soluble carbohydrates between wildtype and RNAi nectaries increase dramatically, the total nectary carbohydrates of wildtype nectary are about three to four times higher than that of RNAi nectary. In addition, we observed the carbohydrate composition changes in the RNAi nectaries (Figure 3.4, panel B). At Stage 9, the nectary carbohydrate ratio, glucose:fructose:sucrose, is around 1:1:1 (molar ratio) in both wildtype and RNAi plants. However, at Stage 12, the carbohydrate ratio, glucose:fructose:sucrose, changed to approximate 1:1:0.5 in RNAi plant nectaries while this ratio is still about 1:1:1 in the wildtype nectaries.

3.3.5 MYB305 knockdown result in altered starch metabolism in nectaries

Because carbohydrates are the catabolic products of nectary starch, we quantified starch contents in the nectaries of wildtype and MYB305 RNAi plants at different development stages. As shown in Figure 3.5, at Stage 3 and Stage 6, no significant difference of starch levels were detected in the wildtype and RNAi line nectaries. From Stage 6 to Stage 9, the starch levels in the wildtype nectaries increased dramatically. In contrast, the RNAi nectaries accumulate much less starch at Stage 9. During normal tobacco flower development, the starch accumulation reaches maximum in nectary at Stage 9 and the nectary starch metabolism will shift from anabolism to catabolism after Stage 9 to provide soluble sugars for nectar secretion and provide substrates for the biosynthesis of important nectary metabolites (Horner et al., 2007; Ren et al., 2007c). As expected, starch content degrades rapidly in the wildtype nectaries after Stage 9, forming a starch anabolism to catabolism transition. In contrast, little difference was observed in the starch levels of RNAi plant nectaries from Stage 9 to Stage 12, which result in loss of such a starch metabolic transition.
Figure 3.5  Nectary starch quantification.

Starch was quantified at Stage 3, 6, 9 and 12 in wildtype and each of the four RNAi lines. Black solid line represent wildtype; blue dashed-line represent RNAi line1; green dashed-line represent RNAi line2; orange dashed-line represent RNAi line 3; red dashed-line represent RNA line 4. Mean ± SD are shown at each data point, n=4.
3.3.6 Starch metabolic genes expression patterns were altered in the MYB305 knockdown plant nectaries

Because starch accumulation in nectaries of the MYB305 RNAi mutants is reduced compared to the wildtype plants, we examined the expression of a number of starch metabolic genes that are known to change during development of the floral nectary. In a previous study, eighteen starch metabolic enzyme genes were examined during nectary development and three different regulatory patterns of gene expression were identified (Ren et al., 2007b). The anabolic genes, ADP-Glucose Pyrophosphorylase small subunit (AGPs) and Starch Synthase 3 (SS3), are highly expressed at early stages (Stage 2 and Stage 6), and down regulated after Stage 9. The catabolic genes, Isoamylase1 (ISA1), α-Amylase (AAM) and β-Amylase (BAM), are only expressed at late development (Stage 9 and later) but not at early stages. The third group of genes, including Starch Branching Enzyme 1 (SBE1) and Stach Phosphorylase (PHO), are expressed throughout nectary development. Because we are interested in the genes whose expressions are changed during the flower development, we focused on the first two groups of genes.

We have shown that myb305 gene is not expressed in the nectary during the early flower development and is dramatically up-regulated at about Stage 6 (Liu et al. 2009). We used quantitative real time RT-PCR to study representative anabolic and catabolic genes from Stage 6 to Stage 12. As shown in Figure 3.6, the myb305 gene expression is significantly increased after Stage 6 in the wildtype plant nectaries. Knockdown of MYB305 result in dramatic decrease of myb305 gene expression in the nectaries of RNAi plants at Stage 8, Stage 10 and Stage 12. To study the effect of MYB305 knockdown on starch metabolic genes expression, we examined expressions of two anabolic genes, AGPs and SS3, and three catabolic genes, AAM, BAM and ISA1. In the wildtype plant nectaries, the two groups of genes show different expression patterns. The anabolic genes, AGPs and SS3, are strongly expressed at early stages (Stage 6 and Stage 8), but are significantly down-regulated at late stages (Stage 12). In contrast, the catabolic genes, AAM,
Figure 3.6  Quantitative real-time RT-PCR detection of starch metabolic gene expressions in the nectaries of wildtype and MYB305 RNAi plants.

Panel A. myb305
Panel B. ADP-Glucose Pyrophosphorylase small subunite (AGPs)
Panel C. Starch synthase 3 (SS3)
Panel D. α-Amylase (AAM)
Panel E. β-Amylase (BAM)
Panel F. Isoamylase 1 (ISA1)

26S rRNA was used as internal control. Four independent biological replicates were used to calculate the mean relative expression value to the 26S expression. Mean + SD are shown at each data point.
BAM and ISA1 are expressed at low levels at early stages (Stage 6 and Stage 8), but are dramatically up-regulated at Stage 12. RNAi knockdown of MYB305 caused different effects on these genes expressions during flower development (Figure 3.6, panels B to F). SS3 gene expression did not show any significant differences between wildtype and RNAi plant nectaries at all four stages that were examined. However, the other anabolic gene, AGPs, is expressed significantly lower in the RNAi plant nectaries at Stage 8. Compared to anabolic genes’ different expression patterns in MYB305 RNAi plant nectaries, the catabolic genes, AAM, BAM and ISA1, are expressed surprisingly similarly in RNAi plant nectaries. RNAi knockdown of MYB305 does not cause significant gene expression change of AAM, BAM and ISA1 between wildtype and RNAi plant nectaries at Stage 6, Stage 8 and Stage 10, but it does result in dramatic decrease of all three genes’ expression at Stage 12. The expression levels of these three catabolic genes in the RNAi nectaries are about 50% lower than that in the wildtype. Thus, we conclude that MYB305 knockdown result in significantly decreased expression of anabolic gene, AGPs, at Stage 8 and catabolic gene, AAM, BAM and ISA1, at Stage 12.

3.3.7 Additional phenotypic changes were observed in petals of MYB305 knockdown plants

We also observed additional phenotypes in the petals of the MYB305 RNAi flowers. The matured Stage 12 wildtype ornamental flower structures are shown in Figure 3.7 panel A. The sepal, floral tube and corolla are on the outside of the ornamental tobacco flower and each flower corolla has five lobes (petals). The petals are on the top of the flowers and we compared wildtype and RNAi petals at different development stages in Figure 3.7 panel B. At Stage 9 and Stage 10, little difference was observed between wildtype and MYB305 RNAi flower petals. From Stage 10 to Stage 11, is usually 12h. During this period, the wildtype flower petals open quickly, while the RNAi flower petals only show slight opening. From Stage 11 to Stage 12, it usually requires another 12h for wildtype flower to reach anthesis. During this period, the wildtype flower petals continue to open and fully expanded to their maximum size, no further petal expansion was observed at 24h after
Figure 3.7  Comparison of wildtype and MYB305 RNAi flowers.

Panel A. Illustration of wildtype ornamental tobacco flower, corolla and petals.

Panel B. Phenotype comparison of wildtype and MYB305 RNAi flowers (top view).
  S9 – Stage 9; S10 – Stage 10; S11 – Stage 11; S12 – Stage 12;
  S12+24h – 24 hours after Stage 12.

Panel C. Limb length measurement of wildtype and MYB305 RNAi flowers.
  Mean ± SD are shown at each bar.
Stage 12. In contrast to the wildtype, the RNAi flower petals expand slowly and do not fully open at Stage 12. After Stage 12, the RNAi plant petals continue to grow and reach their maximum size at ~24h after Stage 12. At this point, the petal color and corolla pattern of RNAi plants are very similar to the wildtype. However, the RNAi plant petals are significantly smaller than wildtype and most of petals can not fully open and reach ~90 degree vertical to flower longitudinal axis. Furthermore, the edge of petals tilt up to outside while the middle areas of petals concave toward insides of flowers, resulting in uneven and crapy petals.

Because the apparent differences of petal size were observed between fully matured wildtype and RNAi flowers, we quantified these differences by measuring the limb length in the middle of the corolla lobe (f) and in the area between corolla lobes (g) (Figure 3.7, panel C). The RNAi plant petals show decreased limb length at both positions, especially in the middle corolla lobe, where the length of RNAi petals is ~50% shorter than the wildtype. Thus, MYB305 knockdown caused abnormal petal development at late stages of tobacco flowers.

3.3.8 Petal expansion is associated with starch metabolism

We have shown that nectary starch accumulation is correlated with MYB305 expression and MYB305 knockout reduces starch accumulation in the nectary. Because MYB305 is also expressed in the petals, we decided to examine starch accumulation in the petals. We used iodine based staining method to monitor starch accumulation in the whole inflorescence. As can be seen in Figure 3.8, panel A, in the wildtype, starch accumulates early in the floral tube and petals of ornamental tobacco flowers (Stage 2, 4 and 6). Iodine staining showed high levels of starch accumulation in floral tube and petals in both wildtype and RNAi lines. At Stage 8 and Stage 9, iodine staining shows high levels of starch in petal, sepal tips and lower floral tube of the wildtype ornamental tobacco flower, while the RNAi flower accumulates most of its starch in the petal. From Stage 8 to Stage 12, starch in the wildtype floral tubes and sepal tips decreases dramatically, but petals maintain high levels of starch at Stage 11 and Stage 12, the starch degrades
Figure 3.8  Petal starch and starch gene expression.

Panel A. Iodine staining of wildtype and MYB305 RNAi flowers. S2 – Stage 2; S4 – Stage 4; S6 – Stage 6; S8 – Stage 8; S9 – Stage 9; S10 – Stage 10; S11 – Stage 11; S12 – Stage 12; S12+24h –24 hours after Stage 12.

Panel B. Petal starch quantification at Stage 10.
Mean ± SD are shown at each bar.

Panel C. AGPs expressions in the petals. 26S rRNA was used as control.
A

<table>
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<th>S6</th>
<th>S8</th>
<th>S9</th>
<th>S10</th>
<th>S11</th>
<th>S12</th>
<th>S12+24h</th>
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<td><img src="image17" alt="Image of RNAi-2 stages" /></td>
<td><img src="image18" alt="Image of RNAi-2 stages" /></td>
</tr>
</tbody>
</table>

B

Starch of corolla lobes (mg/flower)

C

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<tr>
<th>WT</th>
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<th>RNAi-2</th>
</tr>
</thead>
<tbody>
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<td><img src="image20" alt="Image of AGPase" /></td>
</tr>
<tr>
<td>26S</td>
<td><img src="image22" alt="Image of 26S" /></td>
<td><img src="image23" alt="Image of 26S" /></td>
</tr>
</tbody>
</table>
rapidly after Stage 12 and is no longer detectable at ~12 hours after Stage 12. In contrast, the rapid degradation of petal starch in RNAi plants occurred much earlier than wildtype, resulting in significantly lower levels of starch in the petals of RNAi plants than wildtype at Stage 11 and Stage 12. To better understand the starch accumulation in petals, we quantified the petal starch of Stage10 flowers in both wildtype and RNAi plants. As expected, the average petal starch weight is significantly lower in the RNAi flower than in the wildtype ornamental tobacco flowers (Figure 3.8, panel B). Because MYB305 knockdown results in decreased expression of starch anabolic gene AGPs in the nectary (Figure 3.6), and we have shown that myb305 gene is expressed in the petal (Figure 2.1). We want to know whether the AGPs gene expression is decreased in the petals of RNAi plants. Thus, we studied the AGPs expression in petals of Stage 10 flowers using RT-PCR. As shown in the Figure 3.8 panel C, the AGPs expression level was lower in the MYB305 RNAi plant petals than in the wildtype plant petals, which is similar to the AGPs expression pattern in the nectary of RNAi plants.

3.3.9 External supply of carbohydrates can not rescue the petal phenotype in MYB305 knockdown plants

The starch staining test in the RNAi plants indicates that the failure of petal expansion is accompanied with the reduced starch accumulation in the petal. To test whether external supply of carbohydrates can offset the effects caused by decreased petal starch accumulation, we performed phenotype rescue experiments with fully elongated, semi-opened Stage 11 flowers from RNAi plants. The flower buds were incubated in distilled water supplemented with fructose rich, glucose rich, or sucrose rich phloem solutions for 4 days. As shown in Figure 3.9 panel A, the flowers incubated with carbohydrate rich phloem solutions do not differ much from the non-treated and water treated RNAi flowers. To quantify this difference, we measured the limb length in the middle corolla lobes, the results show that there is no significant difference between sugar feeding phloem solutions treated flowers and non-treated flowers. Even if the RNAi flowers were given external carbohydrates, the limb length of RNAi flowers is significantly shorter than
Figure 3.9  Sugar feeding experiment with MYB305 RNAi flowers.

Panel A. Comparison of MYB305 RNAi flowers with different sugar feeding solutions. Non-treated, the flower grown on the MYB305 RNAi line 2 plants; water, the flower treated with water; fructose, the flower treated with fructose feeding solution; glucose, the flower treated with glucose feeding solution; sucrose, the flower treated with sucrose feeding solution; wildtype, the flower grown on the wildtype LxS8 plants.

Panel B. Limb length comparison of wildtype and sugar feeding solution treated flowers. Mean ± SD are shown at each bar.
A

<table>
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<tr>
<th>Non-treated</th>
<th>Water</th>
<th>Fructose</th>
<th>Glucose</th>
<th>Sucrose</th>
<th>Wild-type</th>
</tr>
</thead>
</table>

![Image of flower treatments]

B

![Bar chart showing limb length for different treatments]

Limb length (r) (mm)

![Bar chart with error bars showing limb length comparison]

Non-treated, Water, Fructose, Glucose, Sucrose, Wild-type.
wildtype. Thus we conclude that the expansion growth of petals can not be rescued by external supply of carbohydrates.

Since it has been reported that the starch accumulated in the sepals are correlated with petal expansion in *N. tabacum* cv Xanthi plants (Kwak et al., 2007). We tested the role of the sepals on petal growth by removing the sepals from wildtype ornamental tobacco flowers at Stage 9 and let the flower continue to grow. In the wildtype flowers without sepals, the petals do not show differences from the petals from normal flowers with sepals (data not shown), which indicates the sepal starch might have different roles in the ornamental tobacco plants and Xanthi plants.

### 3.4 Discussion

In earlier work, we identified MYB305 as a transcription activator involved in regulating *Nectarin I* and *Nectarin V* genes expression in the nectary. The MYB305 RNAi knockdown *N. tabacum* cv Xanthi plants show many phenotypes and suggest the broader functions of MYB305, thus we made the transgenic lines of *LxS*-MYB305 in its original *LxS8* (*N. langsdorffii* × *N. sanderae*) ornamental tobacco plants. This study is a continuation of our earlier work focused on the floral phenotypes that are caused by MYB305 knockdown.

#### 3.4.1 The roles of MYB305 in starch metabolism

Ornamental tobacco floral nectaries accumulate high levels of starch beginning at early stages of development and continuing until approximately floral Stage 9 when nectary starch attains peak levels (Ren et al., 2007c). In the final 24 hours before anthesis, nectary starch is rapidly degraded to provide sugars for nectar production (Ren et al., 2007c) as well as substrates for the biosynthesis of important nectary metabolites (Horner et al., 2007). Because the MYB305 RNAi plants show defects in these phenotypes, we focused this study on determining the roles of MYB305 in this context. Therefore, we studied the carbohydrates and starch contents in the RNAi plants at different stages. Our results indicate that in
early stages, prior to expression of MYB305, the starch accumulation is equal in both wildtype and the MYB305 RNAi plant nectaries. However, the starch accumulation is maximum between Stage 6 and Stage 9. Nectar secretion appears to be a mechanistic metabolic process that is triggered by rapid starch hydrolysis. This hydrolysis releases so much free sugars that the cellular osmolarity is dramatically increased. The resulting reduced water potential triggers a massive influx of water into the nectary leading to a building of hydrostatic pressure that is released by fluid secretion through the nectary pore. Because of the reduced starch levels in the RNAi nectaries, hydrolysis of that starch can not provide sufficient soluble sugars to achieve an efficient nectar secretion. Thus lower levels of free sugars are in the Stage 12 nectaries in MYB305 RNAi plants, which is likely the reason that nectar secretion was greatly reduced. Another interesting result is the change of free carbohydrate ratio in the Stage 12 MYB305 RNAi plant nectaries, the ratio of glucose:fructose:sucrose changed from ~1:1:1 to ~1:1:0.5 (molar ratio). The lower percentage of sucrose, the majority carbohydrate in the nectar, indicates the glucoses produced by hydrolysis of starch are being used in places other than sucrose synthesis, which also reflects the deficiency of carbohydrate in the MYB305 RNAi plant nectaries. In addition, an indirect result is the decreased synthesis of β-carotene in the MYB305 RNAi plant nectaries, which has been proposed to be governed by availability of substrate molecules that arise from starch breakdown (Horner et al., 2007; Ren, 2007). Our results that decreased β-carotene synthesis is correlated with starch metabolism supports the conclusion that starch hydrolysis provides precursors for β-carotene biosynthesis.

To study the underlying molecular mechanisms that are responsible for the starch accumulation failures in the nectary, we studied a few highly expressed starch metabolic genes using quantitative real-time RT-PCR. The starch catabolic genes expression patterns are particularly interesting. AAM is an endoamylase that has been found to initiate starch degradation and breakdown long starch molecules on the surface of starch granule of cereal (Smith et al., 2005). In the wildtype plant nectaries, it is expressed at very low levels in early stages (Stage 6 and Stage 8),
but is up-regulated at ~Stage 10 and reaches very high levels at Stage 12 when nectaries is actively secreting nectar. In the RNAi plant nectaries, this up-regulation of AAM gene at Stage 12 is significantly decreased and the expression levels of AAM in RNAi lines is only about 50% of that in wildtype nectaries. BAM is an enzyme capable of cutting soluble linear glucans into small molecules and ISA1 is a debranching enzyme that can remove branches from branched glucans (Smith et al., 2005). Both two enzymes play important roles in starch degradation process and they show similar expression patterns to AAM gene: they are expressed at low levels in early stages and are strongly expressed at Stage 12 in the wildtype nectaries, and their expressions are significantly lower in RNAi plant nectaries than the wildtype at Stage 12. Because all three starch catabolic genes expressions are decreased in the RNAi plant nectaries at Stage 12, it is reasonable to explain that less nectar is produced in the RNAi flowers and less β-carotene is synthesized at anthesis are caused by insufficient soluble carbohydrates produced from degradation of nectary starch. Furthermore, the fact that all three catabolic genes expression are decreased in the RNAi plant nectaries suggests that MYB305 could be involved in controlling starch degradation in broader range via some indirect regulation mechanism. And evaluating other starch degradation genes expressions in MYB305 RNAi plant nectaries is currently an active project in our lab. On the other side, the starch anabolic gene, AGPs and SS3 shows similar expression patterns in wildtype and RNAi plant nectaries. Both genes’ expressions are dramatically decreased at anthesis, indicating starch biosynthesis is no longer a primary job for fully matured nectaries. However, the AGPs gene expression is significantly lower in the MYB305 RNAi plant nectaries than in the wildtype at Stage 8, suggesting that MYB305 may also be involved in positively controlling AGPs gene expression.

3.4.2 Starch and floral organ development

Disrupted starch metabolism occurs in the nectary and petals of MYB305 knockdown plants and resulting in phenotypic changes in both organs.
In the nectary, *myb305* gene starts its expression at ~Stage 6 and the expression continues till anthesis. The phenotypic changes are expected to occur after Stage 6 or even later. Consistent to the expectations, we did observe the altered phenotypes of nectary at Stage 12 in MYB305 RNAi plants. The two apparent phenotypic changes are color and nectar secretion. The color of wildtype matured nectary usually is pumpkin orange, while this color is light yellow in the RNAi plant nectary. The difference of nectary colors is caused by the different levels of β-carotene synthesized in the nectary (Horner et al., 2007). The biosynthesis of β-carotene in the tobacco nectary starts from glucose, which connects the starch degradation pathway with β-carotene synthesis pathway and explains that less β-carotene is synthesized in RNAi plant nectaries is because of insufficient glucose produced from starch degradation. The fewer amount of nectar secretion in RNAi plant secretion is the other nectar phenotype. The major contents in the nectar are soluble sugars, sucrose, glucose and fructose (Ren, 2007). These sugars must reach high levels in the nectary parenchyma cells so that they can provide enough osmotic pressure for nectar secretion. The low levels of starch accumulated in RNAi plant nectaries at early stages result in less soluble sugars produced from starch degradation pathway at late stages, the resulting nectary has less than normal osmolority and can not secret nectar as usual. Both nectary phenotypic changes can be connected to the nectary starch metabolism. Thus we conclude the starch metabolism is crucial for normal nectary development and maturation.

Another phenotype of MYB305 RNAi plants is the failure of petals to expand during anthesis. Petal expansion is believed to be due to cell expansion caused by increasing osmotic pressure, which is due to the degradation of storage carbohydrates and/or the import of sucrose (van Doorn and van Meeteren, 2003). Many young petals have been reported to contain high starch concentrations including *Lilium* (Bieleski *et al.*, 2000b), *Magnolia grandiflora* (Griesel, 1954), *Rosa* species (Ho and Nichols, 1977), *Tradescantia reflexa* (Horie, 1961), and *Turnera ulmifolia* (Ball, 1933). Inhibition of the starch degradation pathway in lily results in
no petal expansion (Bieleski et al., 2000). In the MYB305 RNAi plants, similar petal expansion failure phenotype was found. Because the RNAi plant petals accumulate less starch before anthesis and the starch is consumed rapidly once the flower starts opening, we propose that the depletion of locally accumulated starch causes petal expansion failure in MYB305 RNAi plants.

Uptake of soluble carbohydrates is also found to contribute to petal growth in some plants (van Doorn and van Meeteren, 2003). To determine whether import of sugars is necessary for petal growth, we performed the rescue experiment with RNAi flowers using externally supplied carbohydrates. Our results showed the external supply of carbohydrate does not have significant effects on petal expansion in the RNAi plants. Because transportation of carbohydrates from sepal starch degradation to petal is required for petal limbs growth in N. tabacum cv Xanthi (Kwak et al., 2007). We further tested the role of sepal starch by removing sepals from wildtype ornamental tobacco flowers. The results show no difference between normal grown flowers and flowers with sepals removed, confirming that it is locally accumulated petal starch that contributes most for normal petal expansion. This result also illustrates the existence of subtle difference of regulating flower petal growth in closely related species.

Overall, our results demonstrate that MYB305 plays a crucial role in the development of nectary and petal of ornamental tobacco plants through the starch metabolism.
Chapter 4 Summarization, discussion and future work

4.1 Summarization of previous work

In the previous chapters (Chapter 2 and 3), we showed our progress toward understanding the roles of MYB305 protein in the tobacco plant development. First, we isolated the \textit{myb305} cDNA from \textit{N. langsdorffii} X \textit{N. sanderae} (LxS8). This \textit{LxS-myb305} cDNA contains 579bp nucleotides, which encode a 192 amino acid polypeptide. This gene is strongly expressed in ornamental tobacco flowers with the highest level in the nectary and with lower levels in the ovary, floral tube and petals (Figure 2.1). In the nectary, the \textit{LxS-myb305} gene is up-regulated and starts its robust expression at about Stage 6 (Figure 2.7, Figure 3.5). The \textit{LxS-myb305} gene is the most abundantly expressed transcription factor in nectary and its transcripts account for 1.51\% of total nectary transcripts identified at Stage 12 (61 individual ESTs from a total of 4,023 Stage 12 ESTs = 1.51\% of nectary transcripts identified at anthesis). All of this indicates that MYB305 is critically important for normal flower development, especially nectary glands.

Second, we characterized the \textit{LxS-MYB305} protein. By using the GFP tag, we confirmed that the transcription factor \textit{LxS-MYB305} localized to the nucleus of the tobacco protoplast cells (Figure 2.3). To investigate if \textit{LxS-MYB305} protein is a transcription activator, we utilized a yeast-one-hybrid system to study the effect of \textit{LxS-MYB305} on targeted reporter genes expressions. Our results showed that the \textit{LxS-MYB305} protein is a transcription activator in the yeast and can activate the effector genes expression via its C-terminal domain (Figure 2.5). The C-terminal activation domain of \textit{LxS-MYB305} protein is about 23 amino acids long and has been found to be conservative across several species, including \textit{Antirrhinum majus}, \textit{Arabidopsis thaliana}, \textit{Gerbera hybrida}, and \textit{Pisum sativa} (Figure 2.2 and Figure 2.4). To understand the mechanism of transcription activation, site-directed mutagenesis is used to study the C-terminal activation domain and the acidic amino acids had been found to be responsible for the transcription activation capability (Figure 2.5).
Third, we studied the function of LxS-MYB305 in nectary gene expression. Since the LxS-MYB305 gene is the most abundantly expressed transcription factor in nectary, it’s very intriguing to know if LxS-MYB305 can transcriptionally regulate any of nectary genes. To achieve these goals, we expressed the LxS-MYB305 protein in E.coli and purified this protein by utilized the GST tag (Figure 2.5). This E.coli expressed LxS-MYB305 protein has been found to bind the nec1 and nec5 promoters in vitro at consensus MYB binding sites specifically (Figure 2.6). Then we deleted the MYB binding regions on the nec1 promoter to test if these mutated promoters can still drive the expression of reporter gene expression in nectary (Figure 2.7). Our results suggest that these MYB binding sites on the nec1 promoter are very important for normal nec1 gene expression and deletion of either of MYB binding sites will cause significant decrease of nec1 gene expression. RT-PCR and Western blotting experiments of LxS-MYB305 and nectary genes at different development stages showed that the LxS-myb305 gene is expressed right before nec1 and nec5 genes, which provides the proper time frame for the hypothesis that LxS-MYB305 protein transcriptionally regulates nec1 and nec5 gene expressions (Figure 2.8). Finally, LxS-MYB305 over-expression and RNAi knockdown plants are generated, the nec1 and nec5 gene expressions are increased in the foliages of LxS-MYB305 over-expression plants and decreased in the LxS-MYB305 RNAi tobacco plants (Figure 2.9). Thus, we conclude that the LxS-MYB305 regulates the nec1 and nec5 gene expression in ornamental tobacco.

Fourth, we studied the roles of LxS-MYB305 on flower development and maturation. Since the LxS-myb305 gene is robustly expressed in the nectary, petal and flower tube, we developed the RNAi ornamental tobacco plants to investigate its effect on flower development. Phenotypically, the Stage 12 nectaries of LxS-MYB305 RNAi plants shows light yellow color compared to the pumpkin orange color of wildtype nectaries (Figure 3.1); the RNAi plant nectaries accumulate less β-carotene (Figure 3.2); produce less nectar than the wildtype (Figure 3.3); and the LxS-MYB305 RNAi flower petals are smaller than wildtype and can not expand fully (Figure 3.7). To correlate these phenotypic changes with the MYB305 function,
we analyzed the carbohydrates and starch content in both LxS-MYB305 RNAi and wildtype ornamental tobacco plants. Our results show that the carbohydrate content are lower in RNAi nectary than in the wildtype, the starch accumulation are disrupted in both nectary and petals of RNAi plant flowers before anthesis (Figure 3.3, Figure 3.4, Figure 3.5). In the molecular levels, we found the AGPs expression is significantly lower in the RNAi plant nectary and petals during the starch accumulation process (Figure 3.5, Figure 3.7). In addition, the starch catabolic gene expressions are also altered during the starch hydrolysis process (Figure 3.6). Our results suggest that LxS-MYB305 affect the nectar maturation and petal morphogenesis by controlling starch metabolism.

4.2 Future work

By generating the LxS-MYB305 over-expression and RNAi plants, we got a great opportunity to study the roles of LxS-MYB305 in the plant development. However, there are still some important questions that we need to answer. The first question is where and when the LxS-MYB305 starts its expression. To answer this question, we need to clone the promoter of LxS-MYB305 gene. Once we get the sequence of LxS-myb305 gene promoter, we can fuse this promoter with a reporter gene and then transform the tobacco plant to study where and when the LxS-myb305 gene is expressed. The second question is which gene expressions are affected in the LxS-MYB305 over-expression and RNAi plants. To answer this question, we propose to utilize the Next-GEN Sequencing technology to investigate the global gene expression profiles in the nectary, petal or other organs that LxS-myb305 gene is expressed. The third question is what other phenotypic changes can be caused by over-expression and knockdown LxS-MYB305. To answer this question, we will study the phenotypic changes of the RNAi plants and correlate those phenotypes with published results to find out the connections. For the third question, we already found a particular phenotype that the RNAi plant flower buds abscise earlier than the wildtype. To find out the possible explanations of observed phenotypic changes, we performed some preliminary experiments and showed part of these results in the following section (section 4.3).
4.3 Preliminary results and discussion

The materials and methods of this section are described in Appendix L.

4.3.1 The MYB305 RNAi flowers abscise earlier

Abscission is a physiological progress that enables plant to rid itself of unwanted organs. To test the potential roles of MYB305 during floral abscission, we analyzed the flower separation process in wildtype tobacco plants as well as MYB305 RNAi plants. In the wildtype ornamental tobacco plants, abscission usually occurs a few days after anthesis. Since the abscission is a fast process and flower opening can last for a few days, one particular scene is that all the flowers at different positions from a single stem can stay on the stem and are opening at a certain time (Figure 4.1, panel A and C), though these flowers opened at different time. In contrast to the wildtype flowers, the RNAi flowers abscise much earlier, some flowers abscise within a few days after anthesis, some flowers abscise during anthesis, some flowers abscise even before anthesis. A typical scene of such RNAi tobacco flowers are shown in Figure 4.1, panel B and D, where only the youngest flowers can stay on the top of branch and all elder flowers already abscised from the stem.

4.3.2 The flower abscission zone breaking strength is reduced in MYB305 RNAi plants

To quantify this early abscission phenotype in the RNAi plants, we measured the breaking strength of the abscission zone in the wildtype, MYB305 RNAi knockdown and overexpression Xanthi plants (Figure 4.2). Throughout the flower development stages, no significant breaking strength difference was observed between wildtype and MYB305 overexpression plants. However, the RNAi plant’s breaking strength is significantly lower than wildtype plants at all development stages. The breaking strength of RNAi plants maintain steady until around Stage 10, after which stage the breaking strength decreases dramatically. At Stage 12, the breaking strength of MYB305 RNAi plants is only about 10% of wildtype tobacco plants.
Figure 4.1. Flower abscissions in wildtype and MYB305 knockdown plants

Panel A. Wildtype *N. tabacum* Xanthi plant.

Panel B. MYB305 knockdown *N. tabacum* Xanthi plant.

Panel C. Wildtype ornamental tobacco plants.

Panel D. MYB305 knockdown ornamental tobacco plant.

Some of early abscission sites on the RNAi plants are indicated by red arrows.
Figure 4.2  Breaking strength of abscission zone at different stages.

*N. tabacum* Xanthi plants are used in this research.

Mean + SD are shown in the figure.

Wildtype, MYB305 overexpression and MYB305 RNAi plants are shown in blue, White and purple separately.
4.3.3 The myb305 gene is expressed at abscission zone and correlated with early abscission phenomenon

We have previously shown that the myb305 gene is robustly expressed in the tobacco flowers, but not in the root, stem and leaf. To test whether myb305 is also expressed in the abscission zone, we examined its expression by RT-PCR. As shown in Figure 4.3A, the myb305 gene is weakly expressed in the abscission zone compared to its expression in nectary and petal. To determine if the myb305 gene expression is correlated with the early abscission phenomenon in the MYB305 RNAi plants, we studied myb305 gene expression in the abscission zone of wildtype and RNAi plants (Figure 4.3B). The results showed that myb305 gene expression is decreased in the abscission zones of all the RNAi plants which suggest the correlation between myb305 gene expression and early abscission effect of RNAi plants.

4.3.4 Light is involved in controlling the flower bud abscission

Light has been found to be involved in regulating flower abscission of soybean and many other plants (Heindl and Brun, 1983), which raised our interest if tobacco flower abscission is also affected by light. To test this, we grew the wildtype tobacco plants under different light conditions: one group is grown in the greenhouse, with direct summer sunlight; the other group is grown indoors, avoid direct sunlight. Both groups are given ~16 hour lights and we measured the flower buds abscission rate after Stage 10 to evaluate the light effects. As shown in Figure 4.4, approximate 90% of wildtype flower buds grown under direct sunlight conditions still stay on the plant at the 6th day after Stage 10. In contrast, less than 50% of the wildtype flowers grown shaded from direct sunlight can stay on the plant at the 6th day after Stage 10. These results suggest that light is involved in controlling the flower bud abscission in tobacco plants.
Figure 4.3 RT-PCR of *myb305* gene.

**Panel A.** *myb305* gene is expressed at different tissues of ornamental tobacco.

The RT-PCR is performed using the same amount of total RNA from nectary, petal, sepal, leaf and abscission zone, except the nectary (1:5) band RT-PCR, which started with 20% of total RNA of nectary (1:1). 26S rRNA is used as control.

Cycle number is 18 for 26S rRNA and 22 for *myb305*.

**Panel B.** *myb305* gene expression is decreased at abscission zone of RNAi plants.

26S rRNA and 5 times diluted total RNA from nectary are used as control. The RT-PCR is performed using the same amount of total RNA from the abscission zone. All four MYB305 RNAi ornamental tobacco plants are used in this study.

Cycle number is 18 for 26S rRNA and 25 for *myb305*. 
A. Expression of *myb305* at different tissues

B. Expression of *myb305* in the abscission zone of wild-type (WT) and RNAi plants
Figure 4.4 Flower inabscission rate of tobacco plants

Wildtype, MYB305 overexpression and MYB305 RNAi *Nicotiana tabacum* cv. *Xanthi* plants are used in this study. The method and algorithm of counting and calculating inabscission rate is described in Appendix J and Appendix L. The experiment conditions are described in the results and methods. Day 0 is the day at which flower has been observed to enter Stage 10.
4.3.5 Direct summer sunlight partially rescued the early abscission caused by MYB305 knockdown

The LxS-MYB305 Arabidopsis homolog AtMYB21 is involved in plant light response (Byongchul et al., 2002). Because light is involved in the flower abscission process in tobacco flowers and the fact that flower abscission is correlated with myb305 expression raised question whether light has any effect on flower abscission in MYB305 RNAi plants. To study this, we measured the inabscission rate of MYB305 RNAi flowers under different light conditions: one group is grown in the green house, with summer sunlight; the other group is grown indoors, avoiding direct sunlight. As shown in Figure 4.4, without direct sunlight, the RNAi flowers stay on the plants decrease dramatically after they entered Stage10, less than 10% RNAi flowers can stay on the plants for more than 6 days once they enter Stage10. In contrast, the RNAi flowers grown under direct sunlight showed great improvement, the inabscission rate is ~3 fold higher than that when grown indoor at the 4th, 5th and 6th day after entering Stage10.

4.3.6 MYB305 overexpression plants abscission process is less sensitive to sunlight

The observation that strong sunlight can partially rescue the early abscission effects in the MYB305 RNAi plants raised the question if light have also effect on the inabscission rate of when MYB305 is constitutively expressed. To test this, we analyzed the inabscission rate in the wildtype and MYB305 overexpression plants (Figure 4.4). Our results showed that the inabscission rate is similar between wildtype and MYB305 overexpression plants under strong direct sunlight growth condition. However, the inabscission rate is much higher in the MYB305 overexpression plants than in the wildtype under shaded condition. The abscission process of MYB305 overexpression plants is less sensitive to light, which suggest that light might affect the abscission via regulating myb305 expression.
4.3.7 Differentially expressed genes have been isolated from abscission zone

Since MYB305 is a transcription activator and its expression is directly linked to abscission, we sought to identify those genes whose expressions were altered in the abscission zone of MYB305 RNAi plants. To achieve this, we utilized the differential display technology to discover these genes. The abscission zone samples are chosen from Stage 9 wildtype abscission zone and Stage12 wildtype abscission zone and Stage12 MYB305 RNAi abscission zone. The results are shown in Appendix K and 44 bands were identified as differentially expressed in the abscission zone. We reamplified all of these bands and cloned five of these bands into pGEM-T vectors. The sequences of these five cDNAs and their preliminary blast analysis are listed in Table 4.1. As we can see, these genes are not well studied and their putative functions are obscure, suggesting there is a lot more work need to be done to discovery roles of these genes in the abscission and plant development.

4.3.8 Progress toward cloning LxS-myb305 gene

To better understand the function of myb305 gene, we tried to clone the LxS-myb305 gene, especially the promoter region, using different methods. The first approach we tried is screening genomic DNA library of Nicotiana plumbaginifolia packed in the lambda DNA by hybridation with labeled probes. Because the 5 prime end cDNA of LxS-myb305 gene encoding the MYB DNA binding domain is highly homologous to other MYB family members, it is not advisable to design the probes in this region. Because the EcoR I site is between the cDNAs encoding the MYB DNA binding domain and activation domain, and the fact that the genomic DNA library is prepared by cutting genomic DNA with EcoR I enzyme, it is also not advisable to design the probe within the cDNA that encodes activation domain for screening library. Thus, the genomic library screening method is not practically useful in our case. The second method we tried is based on bioinformatic approach. We collected as much as possible LxS-myb305 related genomic DNA sequences from different Nicotiana plants and assembled these sequences together to predict the structure of LxS-myb305 gene. A few primers
were designed in conserved DNA region and were used to amplify the genomic DNA of ornamental tobacco plants. By using this method, a ~300 bp long promoter region was cloned. Its sequence is listed in Appendix M along with the primers used in this research and the predicted \textit{LxS-myb305} gene structure. Because of the cloning of ~300 bp long \textit{LxS-myb305} gene promoter, we tried to utilize this information to clone the full length \textit{LxS-myb305} promoter. The methods we tested are tail PCR and emulsion PCR. However, we could not amplify the full length \textit{LxS-myb305} gene promoter by using the above two methods with current set of primers (Appendix M) and this project is an ongoing study in our lab.
<table>
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<th>Sequence</th>
<th>Putative functions by blast results (Reference sequences, GenBank)</th>
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<td>FDD02</td>
<td>CGACTGTACATAAGCTAAACTTTAGTACCTTTGCTTCCGTACATGGAACCTCAACATTGGTACAGTCTGACCTTGCAGTATCATTCTCGTACATTTTCTGTACATGGAACCTCAACATTGGTACAGTCTGACCTTGCAGTATCATTCTCGTCATCACTTCTGCACTTTTAGCTTTAAAATACTTCTTACATTTTAGCTTTAATAAAAAAAAAAAA</td>
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<td>FDD09</td>
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<td>Respond to hormone by blastn Ref</td>
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<tr>
<td>FDD14</td>
<td>AGTAGGGCAAGAAAATGTTTGTGTTTACCTCTTTGCTTCCGTACATGGAACCTCAACATTGGTACAGTCTGACCTTGCAGTATCATTCTCGTACATTTTCTGTACATGGAACCTCAACATTGGTACAGTCTGACCTTGCAGTATCATTCTCGTACATTTTCTGTACATGGAACCTCAACATTGGTACAGTCTGACCTTGCAGTATCATTCTCGTCATCACTTCTGCACTTTTAGCTTTAAAATACTTCTTACATTTTAGCTTTAATAAAAAAAAAAAA</td>
<td>Found in leaves of N.tabacum by blastn Ref</td>
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<td>Relate to cold-dark stress by blastn AM845384.1 Cold overnight library N.tabacum mRNA,6e-26</td>
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<td>FDD19</td>
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<td>Relate to stress response by blastn gb</td>
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**Appendix A. Oligonucleotides used in these studies.**

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<td>MYBGST2</td>
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<td><strong>Transcriptional activation assays, over-expression and RNAi transformation</strong></td>
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<td>RT-PCR and quantitative RT-PCR of chi</td>
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<td>Sequence</td>
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<td>RT-PCR and quantitative RT-PCR of ( \text{26S} ) amplifies a 235 bp amplicon</td>
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<tr>
<td>18S-F</td>
<td>5'-TTGCTATGGGCGTGTTACTG-3'</td>
<td>RT-PCR and quantitative RT-PCR of ( \text{26S} ) amplifies a 235 bp amplicon</td>
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<td>18S-R</td>
<td>5'-TCGGCAAAGCTATAACTCCG-3'</td>
<td>RT-PCR and quantitative RT-PCR of ( \text{26S} ) amplifies a 235 bp amplicon</td>
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<td>RT-agps-F</td>
<td>5'-GATTTTAGCTTCTAGGAGCAGTACG-3'</td>
<td>RT-PCR and quantitative RT-PCR of ( \text{AGPase} ) small subunite amplifies a 125 bp amplicon</td>
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<td>RT-agps-R</td>
<td>5'-CAGTTCTTGTACACAACCTTCACC-3'</td>
<td>RT-PCR and quantitative RT-PCR of ( \text{AGPase} ) small subunite amplifies a 125 bp amplicon</td>
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<td>5'-ATGAGACCTACTGGAAGGGTGTTAATGTGC-3'</td>
<td>RT-PCR and quantitative RT-PCR of starch synthase 1 amplifies a 88 bp amplicon</td>
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<td>RT-ss1-R</td>
<td>5'-AAATCCAATCAGCGAGAATCG-3'</td>
<td>RT-PCR and quantitative RT-PCR of starch synthase 1 amplifies a 88 bp amplicon</td>
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<td>5'-ATGAGACCTACTGGAAGGGTGTTAATGTGC-3'</td>
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<td>RT-gbss-F</td>
<td>5'-ATCTGAAATGCGAAGGGTGTTAATGTGC-3'</td>
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<td>RT-gbss-R</td>
<td>5'-GCCTTTACATCCTCAGCAGAATCG-3'</td>
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<td>RT-iso1-F</td>
<td>5'-TTGAGATCCCTTATGCCAAGGTTGCA-3'</td>
<td>RT-PCR and quantitative RT-PCR of starch isoamylase 1 amplifies a 135 bp amplicon</td>
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<td>RT-iso1-R</td>
<td>5'-TAGTGCTCTTTCCTCAGCAGAATCG-3'</td>
<td>RT-PCR and quantitative RT-PCR of starch isoamylase 1 amplifies a 135 bp amplicon</td>
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<td>5'-AACGAGATGCTTCTGCAAGGTTGCA-3'</td>
<td>RT-PCR and quantitative RT-PCR of starch isoamylase 3 amplifies a 279 bp amplicon</td>
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<td>5'-TTGAAAGGTTATGGAGAGGAGGAAATCGGAG-3'</td>
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<td>RT-bam-F</td>
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<td>RT-gap2-F</td>
<td>5'-TTGAAGGGTGGATGCCAAGAAAATCGGAG-3'</td>
<td>RT-PCR of GADPH amplifies a 161 bp amplicon</td>
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<td>RT-gap2-R</td>
<td>5'-CATTGTAGACCTTTTGGAGAGGACGGAATCGC-3'</td>
<td>RT-PCR of GADPH amplifies a 161 bp amplicon</td>
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**Mobility shift assays**

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<td>5'-AAGCCTAAAATACCTAACAGAGCTAACA-3'</td>
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<td>N1P1-LOWER</td>
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<td>N1C1-UPPER</td>
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<td>5’-TAAAATAATTCACCTAAACTTCAAGATA-3’</td>
<td>labeled and unlabeled N5P dsDNA probe for mobility shift assays</td>
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<td><strong>N5PCmptF</strong></td>
<td>5’-TAAAATAATTTTTTTTTTTTTTTTTTTTAAATTATTT-3’</td>
<td>N5C dsDNA probe used as a competitor in the mobility shift assays</td>
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<td><strong>N5PCmptR</strong></td>
<td>5’-TATCTTGAAAGTTAGGTAATTATTTTA-3’</td>
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**necl promoter analysis**

| **p454-1** | 5’-ATATTGGGCCCTTAGTTGTGTCCACAAATCAC-3’ | PCR - necl promoter 5’ end (-1216) – ApaI site |
| **p454-2** | 5’-CTATTCCCGGTAAGGACGTGTCCAGATTGT-3’ | PCR upstream of S1 site (-898) – SacII site |
| **p454-3** | 5’-CTATTCCCGGGAATCCGACGAAACTCCATT-3’ | PCR downstream of S1 site (-892) - SacII site |
| **p454-4** | 5’-TAACGACTAGTCAGGTCAATTCCAATACAA-3’ | PCR upstream of S1 site (-) - SpeI site |
| **p454-5** | 5’-TAACGACTAGTCAGGTCAATTCCAATACAA-3’ | PCR downstream of S1 site (-) - SpeI site |
| **p454-6** | 5’-AATCGGAGCTC AAGGAGAAG AAGAACTCG-3’ | PCR – necl promoter 3’ end (+50) – SacI site |
| **p454-7** | 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGTTGTCCACAAATCAC-3’ | To clone the original and mutated necl promoters into Gateway™ entry vector pDONR/Zeo |
| **p454-8** | 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTCAAGGAAAGAAACTCG-3’ |  |

**Abscission zone differential displays**

| **RH-T11G** | 5’-R-AAGCTTTTTTTTTTTTTGTG-3’ | Fluorescent (Rhodamine) labeled anchor primers |
| **RH-T11A** | 5’-R-AAGCTTTTTTTTTTTTTTA-3’ |  |
| **RH-T11C** | 5’-R-AAGCTTTTTTTTTTTTTTC-3’ |  |
| **H-AP1** | 5’-AAGCTTGGATTC-3’ | Unlabeled random primers |
| **H-AP2** | 5’-AAGCTTGGACTG-3’ |  |
| **H-AP3** | 5’-AAGCTTGGATTC-3’ |  |
| **H-AP4** | 5’-AAGCTTGGATTC-3’ |  |
| **H-AP5** | 5’-AAGCTTGGATTC-3’ |  |
| **H-AP6** | 5’-AAGCTTGGACAT-3’ |  |
| **H-AP7** | 5’-AAGCTTGGACAT-3’ |  |
| **H-AP8** | 5’-AAGCTTGGACAT-3’ |  |
Appendix B. Alignment of MYB region of LxS-MYB305 protein with other MYB proteins
### Alignment

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<th>An-MYB105</th>
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<th>Fe-MYB26</th>
<th>Pe-MYB21</th>
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### GenBank accession number

LmS-MYB305 (gene 1) (EU111679); Fe-MYB26 [CAA71992]; An-MYB305 [DAD87008]; An-MYB340 (F81391); An-MYB88 (F81396); At-MYB21 [NP_159418 [At3g27810]]; At-MYB24 (NP_159851 [At5g46350]); At-MYB57 (NP_156802 [At3g15301]).
Appendix D. Additional phylogenetic analysis of LxS-MYB305

GenBank accession number

LxS-MYB305 gene 1 (U114679); Ps-MYB26 (CAA71992); At-MYB21 (NP_189418 [At3g27610]); At-MYB24 (NP_190851 [At5g03500]); At-MYB57 (NP_186802 [At3g01530]);
Appendix E. Yeast-2-hybrid test of homodimerization with LxS-MYB305

Panel A: Prey and Bait constructs used in this experiment;
Panel B: Serial-dilution growth test result on -Uracil medium, positive and negative controls are shown as illustrated.
Appendix F. Wild-type and MYB305 over-expression plants

Phenotypes of 3-month old wild-type and MYB305 over-expression plants.
Panel A. Wild-type *N. tabacum* cv Xanthi plants.
Panel B. MYB305 over-expression *N. tabacum* cv Xanthi plant #3.
Panel C. MYB305 over-expression *N. tabacum* cv Xanthi plant #4.
Panel D. Leaf from wild-type *N. tabacum* cv Xanthi plants.
Panel E. Leaf from MYB305 over-expression *N. tabacum* cv Xanthi plant #3.
Panel F. Leaf from MYB305 over-expression *N. tabacum* cv Xanthi plant #4.
Appendix G. RT-PCR analysis in the foliage of MYB305 over-expression plants

RT-PCR of interested genes in the foliage of wild-type & MYB305 over-expression N.tabacum cv Xanthi plants.
Appendix H. Length of flower organs at anthesis

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<th>Wildtype</th>
<th>MYB305 RNAi</th>
<th>MYB305 OX</th>
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<tr>
<td>shortest anther length</td>
<td>33.3 ± 1.3</td>
<td>25.9 ± 2.5</td>
<td>30.2 ± 1.4</td>
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<td>longest anther length</td>
<td>38.3 ± 1.6</td>
<td>31.1 ± 1.8</td>
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<tr>
<td>style length</td>
<td>35.4 ± 0.9</td>
<td>34.1 ± 1.5</td>
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<td>floral tube length</td>
<td>37.7 ± 1.2</td>
<td>35.0 ± 1.4</td>
<td>24.4 ± 1.2</td>
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<tr>
<td>n</td>
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<td>30</td>
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Student’s T-test comparisons of each combination:
1. Shortest anther length: WT > OX > RNAi P<0.01 for each pair comparison
2. Longest anther length: WT > OX > RNAi P<0.01 for each pair comparison
3. Style length: WT > RNAi >OX P<0.01 for each pair comparison

Method:
Floral parts were dissected from flowers and anther and style length was measured. Because each flower contains one short and four long anthers, both the shortest and longest anthers were measured. All four RNAi plants were pooled together in the RNAi knockdown study. Similarly, the OX3 and OX4 lines were pooled together in the over-expression study.
Appendix I. Illustration of measuring abscission zone breaking force

slowly fill the tube with water using transfer pipette until flower bud breaks.

measure weight
The first data on each column is the number of flowers observed to enter Stage 10 at the day indicated at the top of column. This date is recorded as day 0. The number of abscised flowers is recorded daily after day 0 for 6 days. If the remaining flowers do not abscise after day 6, the number of these flowers will be put into day 7. The total number of abscised flowers at 1\textsuperscript{st}, 2\textsuperscript{nd}, 3\textsuperscript{rd}, 4\textsuperscript{th}, 5\textsuperscript{th}, 6\textsuperscript{th} and 7\textsuperscript{th} day are calculated separately to measure the inabscission rate.

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</table>
Appendix K. Abscission zone differential display results
Appendix L. Materials and methods of section 4.3

Materials

Unless otherwise noted, the materials used in these studies were obtained from either Fisher Chemical Co (www.fishersci.com) or Sigma Chemical Co (www.sigmaaldrich.com) and were of the highest quality available.

Plants

The *N. langsdorffii* X *N. sanderae* (*LxS8*) ornamental tobacco plants, the *LxS*-MYB305 RNAi *LxS8* plants, the *Nicotiana tabacum* cv. *Xanthi* plants and *LxS*-MYB305 RNAi Xanthi plants are used in these studies, the growth condition of plants were described in chapter 2 and chapter 3.

Measurement of breaking force

Abscission force was quantitatively measured using lab-made recording abscissor similar to the one described by (Craker and Abeles, 1969). Basically, the flower was held inverted stationary by clamping the stem that is connected to the bottom end of flower bud, the other clamp that linked to a BD Falcon 50ml conical tube was used to clamp the flower tube. Distilled water is gradually filled into the conical tube until the flower bud abscised from the stem. The total weight of conical tube, water and clamps are recorded and used to describe abscission force. A illustration of this device is shown in Appendix H.

RT-PCR

The tobacco tissues were collected and frozen in liquid nitrogen; RNAs were then purified using the TRIzol Reagent method (www.invitrogen.com) according to the manufacturer's instructions. The first strand cDNAs were made using the Eppendorf cMaster RT PCR System. All the primers used in this study are given in Appendix A. 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 photographed immediately after running.

**Abscission observation under different conditions**

To study how long the flower buds can stay on the stem once they enter anthesis period, the day that individual tobacco flower bud enters Stage9 was recorded and that flower bud was marked. Then the day that earlier marked flower bud abscised was recorded and the time span between abscission and early anthesis was calculated to investigate the flower bud retaining time. To facilitate the recording work, a special format work table was used and a sample of this table is shown in Appendix I

**Differential display**

Wild type LxS8 plant and MYB305 RNAi plant with the most significant phenotype (#3) were used in this research. The primers used in this research are composed of two parts: poly T anchor primers: RH-T11G, RH-T11A (R represent Rhodamine labeled); and arbitrary primers: H-AP1, H-AP2, H-AP3, H-AP4, H-AP5, H-AP6, H-AP7, H-AP8. The sequences of these primers are listed in Appendix A and sixteen pairs of primer are used in this research. The RNA from Stage 9 wildtype, Stage12 wildtype and Stage12 RNAi plants abscission zone were extracted using TRIzol Reagent method (www.invitrogen.com) according to the manufacturer’s instructions. RT-PCR was performed used the RNAspectraRed kit#1 from GenHunter (GenHunter.com). The products of RT-PCR reaction were loaded onto a 24cm long 1mm thick 8% denaturing polyacrylamide sequencing gel. Electrophoresis was performed at 300 volt and the gel was imaged by Typhoon 8600 imaging system (GE healthcare), excitation at 532nm, emission filter 580BP30. The identified band was further cut from the gel, PCR amplified, cloned into pGEM-T vector and sequenced at Iowa State University DNA facility.
Appendix M. Summary of myb305 gene cloning

1. Predicted LxS-myb305 gene structure.

![Gene Structure Diagram]

2. Part of LxS-myb305 gene sequence that had been cloned.

<table>
<thead>
<tr>
<th>Promoter region</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter region</td>
<td>gatcacatcaatagctgtcatgtatgcctgcataagttatttcctattacaagcctttctttaaagatgcttattaccaag tcttcattaagagattatagcttattatgttattatctttataaagacagtta ggtttaaagctctctttgtctgcaaatcttaaagactaattactagtagcata ttttattattagcaccctgttataaatatctgccaaccacattagttcttgc tccaaga</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intron 1</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intron 1</td>
<td>attccttagctaatctggaatttttcttcctttccctttgc</td>
</tr>
<tr>
<td>Intron 1</td>
<td>agtaaacaacccctagatatatattatagctataacactttttatagtct</td>
</tr>
</tbody>
</table>

3. Primers used in tail PCR research.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mtail-1</td>
<td>GCGACATAAGAGCTTTTAACCTAAGTGC</td>
<td>In reverse direction. LxS-myb305 gene specific primer, used for tail PCR</td>
</tr>
<tr>
<td>Mtail-2</td>
<td>CTTGCTTTATTATAAAATCATATAAATCTTACATATAACT</td>
<td></td>
</tr>
<tr>
<td>Mtail-3</td>
<td>GACTTAAAACATTCAGGCATACATGAG</td>
<td></td>
</tr>
<tr>
<td>AD1</td>
<td>NGTCGASWGANAWGAA</td>
<td>Degenerate arbitrary primers used in tail PCR</td>
</tr>
<tr>
<td>AD2</td>
<td>TGWGNAGSANCASAGA</td>
<td></td>
</tr>
<tr>
<td>AD3</td>
<td>AGWGNAGWANCAWAGG</td>
<td></td>
</tr>
<tr>
<td>AD4</td>
<td>STTGNTASTNCTNTGCA</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>A - Adenosine</th>
<th>G - Guanosine</th>
<th>C - Cytidine</th>
<th>T - Thymidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>R - A or G</td>
<td>Y - C or T</td>
<td>K - G or T</td>
<td>M - A or C</td>
</tr>
<tr>
<td>S - G or C</td>
<td>W - A or T</td>
<td>B - C, G or T</td>
<td>D - A, G or T</td>
</tr>
<tr>
<td>H - A, C or T</td>
<td>V - A, C or G</td>
<td>N - any base</td>
<td>I - Inosine</td>
</tr>
</tbody>
</table>
4. Program used for Tail PCR

**TAIL 1° REACTION PROGRAM:** Control Method: CALCULATED

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Stage/Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4°C</td>
<td>2 min.</td>
<td>Pre-Run stage</td>
</tr>
<tr>
<td>2</td>
<td>94°C</td>
<td>2 min.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>94°C</td>
<td>30 sec.</td>
<td>5 high-stringency cycles</td>
</tr>
<tr>
<td>4</td>
<td>62°C</td>
<td>1 min.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>72°C</td>
<td>2 min. 30 sec.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Go to step-3 for 4 more cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>94°C</td>
<td>30 sec.</td>
<td>1 low-stringency cycle</td>
</tr>
<tr>
<td>8</td>
<td>25°C</td>
<td>3 min.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Ramp for 72°C at 0.2°C/sec</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>2 min. 30 sec.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>94°C</td>
<td>20 sec.</td>
<td>2 high-stringency cycles</td>
</tr>
<tr>
<td>11</td>
<td>65°C</td>
<td>1 min.</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>72°C</td>
<td>2 min. 30 sec.</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>94°C</td>
<td>20 sec.</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>65°C</td>
<td>1 min.</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>72°C</td>
<td>2 min. 30 sec.</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>94°C</td>
<td>20 sec.</td>
<td>1 medium-stringency cycle</td>
</tr>
<tr>
<td>17</td>
<td>44°C</td>
<td>1 min.</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>72°C</td>
<td>2 min. 30 sec.</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Go to step-10, for 14 more cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>72°C</td>
<td>5 min.</td>
<td>15 TAIL cycles</td>
</tr>
<tr>
<td>21</td>
<td>4°C</td>
<td>forever</td>
<td>Finish and Post-Run Stage</td>
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<tr>
<td>22</td>
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**TAIL 2° REACTION PROGRAM:** Control Method: CALCULATED

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</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C</td>
<td>1 min.</td>
<td>2 high-stringency cycles</td>
</tr>
<tr>
<td>2</td>
<td>94°C</td>
<td>20 sec.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>64°C</td>
<td>1 min.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>72°C</td>
<td>2 min. 30 sec.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>94°C</td>
<td>20 sec.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>64°C</td>
<td>1 min.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>72°C</td>
<td>2 min. 30 sec.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>94°C</td>
<td>20 sec.</td>
<td>1 medium-stringency cycle</td>
</tr>
<tr>
<td>9</td>
<td>44°C</td>
<td>1 min.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>72°C</td>
<td>2 min. 30 sec.</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Go to step-2, for 11 more cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>72°C</td>
<td>5 min.</td>
<td>12 TAIL cycles</td>
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<tr>
<td>13</td>
<td>4°C</td>
<td>forever</td>
<td>Finish and Post-Run Stage</td>
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<tr>
<td>14</td>
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**TAIL 3° REACTION PROGRAM:** Control Method: CALCULATED

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<td>1</td>
<td>94°C</td>
<td>1 min.</td>
<td>PCR: 20 cycles of medium-stringency</td>
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<td>2</td>
<td>94°C</td>
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<td>3</td>
<td>44°C</td>
<td>1 min.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>72°C</td>
<td>2 min. 30 sec.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Go to step-2, for 20 more cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>72°C</td>
<td>5 min.</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4°C</td>
<td>forever</td>
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</tr>
<tr>
<td>8</td>
<td>END</td>
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References


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Darwin, C. (1890). The Various Contrivances by which Orchids are Fertilised by Insects (2nd edition)., (London, UK.: John Murray.).


