Functional Analysis of the MYB75 Transcription Factor as a Regulator of Secondary Cell Wall Formation in Arabidopsis

by

Apurva Bhargava

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Botany)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

December 2010

ABSTRACT

Deposition of lignified secondary cell walls in plants is spatially and temporally regulated by transcription factors (TFs). MYB75 is a known regulator of the anthocyanin branch of the phenylpropanoid pathway in Arabidopsis. It was established in this work that this TF also regulates secondary cell wall formation in Arabidopsis. A loss-of-function mutation in MYB75 (*myb75-1*) results in increased cell wall thickness in xylary and interfascicular fibers within the inflorescence stem. Transcript profiles from the *myb75-1* inflorescence stem revealed marked up-regulation in the expression of a suite of genes associated with lignin biosynthesis and cellulose deposition, as well as genes encoding cell wall-modifying proteins and genes involved in photosynthesis and carbon assimilation. These patterns suggest that MYB75 acts as a repressor of the lignin branch of the phenylpropanoid pathway in the Arabidopsis inflorescence stem as part of a broader role in integrating the metabolic flux through different pathways. Secondary cell wall-associated and potential transcription factors may interact together and form a multi-protein complex to regulate the formation of the secondary cell wall. I identified many positive interactions among the known and putative secondary cell wall formation-regulating TFs, using yeast two-hybrid assays. The KNAT7-MYB75 interaction detected in these assays was selected for detailed study, since both of these TF's individually have been shown to regulate some aspects of secondary cell wall formation in Arabidopsis. The KNAT7-MYB75 interaction was confirmed *in vivo* in an Arabidopsis protoplast system, and the major domains involved in these protein-protein interactions were identified. This interaction may also be involved in regulation of secondary cell wall formation during Arabidopsis seed coat development. This was evident through phenotypic analysis of single and double loss-of-function mutants using molecular and histological analyses that revealed epidermal cell wall defects. However, MYB75, unlike KNAT7, appears to be a specific regulator of secondary cell wall formation in the seed coat, with no role in mucilage biosynthesis. I propose that a MYB75-KNAT7-involving protein complex could be participating in regulation of secondary cell wall biosynthesis in the developing seed coat, based on identified protein-protein interactions among MYB75, KNAT7 and other transcription factors known to influence seed coat properties.

PREFACE

This thesis is written as a series of manuscripts (three) with the intent of publication in peer-reviewed journals. **Chapter 1** provides the thesis framework, literature review and research objectives, while **Chapter 5** discusses the salient findings and the future directions. All co-authors in this thesis declare no conflicts of interest.

Chapter 2

All experiments in this chapter were designed and executed by me. Hardy Hall did the microarray data analysis.

Chapter 3

I designed and executed all the experiments in this chapter. Dr Shucai Wang contributed to protoplast isolation.

A version of chapter 2 and part of chapter 3 has been published:

Bhargava, A., Mansfield, S.D., Hall, H.C., Douglas, C.J. and Ellis, B.E.E. (2010). MYB75 functions in regulation of secondary cell wall formation in the Arabidopsis inflorescence stem (Plant Physiol. Published on August 31, 2010; 10.1104/pp.110.162735)

I conducted all the research and wrote most of the manuscript. Hardy Hall assisted in microarray data analysis

Chapter 4

Dr. Abdul Ahad provided cDNA samples of seed coat regulators in different stages, contributed to ruthenium red staining and qRT-PCR. I designed and executed all other experiments in this chapter.

A part of chapter 3 and version of chapter 4 is in process of manuscript preparation

Bhargava, A., Ahad, A., Wang, S., Mansfield, S.D., Haughn, G., Douglas, C. and Ellis, B.E.E. MYB75-KNAT7 interaction involvement in regulating secondary cell wall deposition in Arabidopsis inflorescence stem and seed coat (In preparation).

I designed and conducted all the research and prepared the manuscript.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3AT	3-amino-1, 2, 4-triazole
AP2/EREBP	APETELA2/ETHYLENE RESPONSE ELEMENT BINDING PROTEIN
BiFC	Bimolecular fluorescence complementation
bp	DNA base pairs
CaMV	Cauliflower Mosaic Virus
CesA	Cellulose synthase
cDNA	Complementary DNA reverse transcribed from messenger RNA
ChIP	Chromatin immunoprecipitation
DNA	Deoxyribonucleic acid
GD	Gal4 DNA binding domain
GR	Glucocorticoid receptor
GUS	β-glucuronidase
HIS	Histidine
irx	Irregular xylem
KNAT	Knotted 1-like (protein/gene) of *Arabidopsis thaliana*
KNOX	Knotted 1-like homeobox (protein/gene)
LB	Luria-Broth
LEU	Leucine
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time RT-PCR
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Reverse transcription
SC	Synthetic complete
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TF	Transcription factor
TRP	Tryptophan
URA	Uracil
WT	Wild-type
X-gluc	5-bromo-4-chloro-3-indoyl-β-D-glucuronide
Y2H	Yeast two hybrid
Y3H	Yeast three hybrid
YFP	Yellow fluorescent protein

ACKNOWLEDGEMENTS

This dissertation would not have been possible without the guidance and the help of several individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study.

First and foremost, I am heartily thankful to my supervisor, Dr. Brian Ellis, whose encouragement, guidance and support from the initial to the final level of this work enabled me to develop a very different outlook to scientific thinking. He provided me with a platform to learn excellent science. Special thanks to my co-supervisor Dr. Carl Douglas for constant support, help and his input in this work. I would also like to thank my committee members, Dr. Steven Lund and Dr. Xin Li, for their constructive contribution into this research project.

Thanks to all the past and present members of Ellis and Douglas laboratory especially QingNing Zeng, Jin Suk Lee, Hardy Hall, Ankit Walia, Jia Cheng, Doris Wong, Adrienne Nye, Dr. Mathias Schuetz, Dr. Albert Cairo, Rebecca Smith, Teagan Quilichini, Eryang Li, Yuanyuan Liu, Dr. Michael Friedman, SungSoo Kim and Dr. Etienne Grienenberger. Special thanks to Dr. Santokh Singh with whom, as a teaching assistant, I learned and taught Cell Biology tutorials in summers to the undergraduate students. Thanks to Veronica Oxtoby and Judy Heyes for all their help. I would like to thank Drs. Vicki Maloney, Andrew Robinson and Alex Skybe (Department of Wood Science, UBC) for their input in chemical analyses, Brad Ross and the UBC Bioimaging facility for TEM support, Dr Shucai Wang (UBC) for help with protoplast transformation, Dr. Abdul Ahad (UBC) for seed coat analysis, Hardy Hall (UBC) for microarray data analysis, Dr. A.M. Lloyd (University of Texas at Austin) for kindly providing MYB75pro::GUS seeds, the Arabidopsis Biological Resource Center for *pap1-D* seeds, RIKEN (Yokohama, Japan) for *myb75-1* seeds. I gratefully acknowledge Dr Joachim Uhrig (Botanical Institute, Universita¨t Ko¨ln) for the kind gift of pBatTL vectors used for BiFC assays.

Thanks to my mother (Veena Bhargava) for drawing the cartoons (Figure 1.1 and Figure 1.2) and all her love and belief in me. I am grateful to all my family members for their support and encouragement. Final thanks go to my wife, Megha, for her love, constant encouragement, positive attitude and emotional and moral support in everything I do. She was strength behind successful completion of this work. In the end, special thanks to my son Vihaan, who was a source of energy in this endeavor.

DEDICATION

To My Parents……

Chapter 1. General introduction

1.1 PLANT CELL WALL

The majority of both plant and animal cells are composed of a cytoplasm bounded by a plasma membrane but specifically in plants, individual cells are surrounded by a rigid wall (Raven, et al., 1999). The wall is formed outside of the plant cell plasma membrane and is a part of the apoplast. Cell walls are a defining feature of plants and have many fundamental roles during growth and development. They play a central role in determining the mechanical properties of all organs and are critical for wide range of cell functions including cell expansion and adhesion. Plant cell walls are the outermost barriers against biotic and abiotic stresses that sessile plants have to face. Being rich in cellulose and lignin, cell walls represent an immense carbon sink. In addition, cell walls are of commercial importance for humans. They not only are source of fibers but are raw material for textile, paper, lumber and biofuels.

The cell wall is an intricate and continuously changing structure made up of various polymers, including cellulose, hemicelluloses, pectins, lignin and glycans. It is highly dynamic throughout development and undergoes modulations in composition and configuration in response to functional requirements. Structurally and functionally, plant cell walls can be divided into two important categories: the primary cell wall and the secondary cell wall (Figure 1.1).

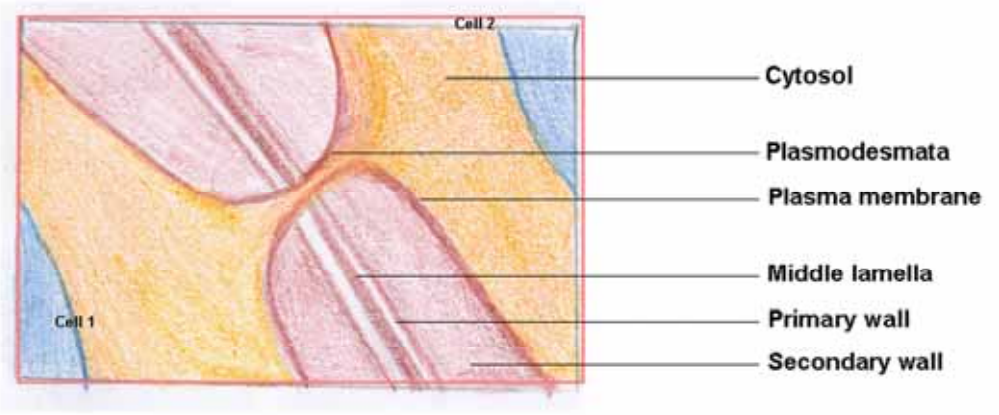


Figure 1.1. Plant cell wall. Diagram of adjacent plant cells connected through their adherent cell walls. Both primary and secondary walls separate the two cells but still allow direct cell-cell communication through plasmodesmata. (Drawing by V. Bhargava, used with permission)

1.1.1 Primary cell wall

The first cell walls that are formed in growing plant cells are called primary cell walls and are thin, strong and pliant. The extracellular layers of this primary wall are composed of cellulose microfibrils, which are surrounded by a hydrated matrix consisting of complex polysaccharides (mostly hemicellulose and pectin) and a small amount of structural proteins, with an overall composition of approximately 90% polysaccharide and 10% glycoproteins. In addition, some modified cell wall constituents (polysaccharides) are also part of the primary cell walls (O'Neill and York, 2003). Cellulose, a linear polymer of β-4-linked D-glucosyl residues, constitutes about 20%-30% of primary cell walls. The remaining polysaccharides are exceedingly complex molecules, none of whose structures have been completely defined. In *Arabidopsis thaliana* (referred to as 'Arabidopsis' in this work), membrane complexes that contain cellulose synthases, encoded by three *CESA* genes (*CesA3, CesA5* and *CesA6*) are responsible for the synthesis of primary wall cellulose microfibrils (Desprez, et al., 2007). In addition, *CESA2, CESA5* and *CESA9* are partially functionally redundant with *CESA6* and also play role in primary cell wall formation at different stages of growth (Desprez, et al., 2007; Persson, et al., 2005).

Xyloglucan (XG) is a hemicellulose polymer that cross-links cellulose microfibrils in primary cell walls. Since primary cell wall expansion is dependent on disassociation of the cellulose microfibrils, many enzymes have been assigned to the loosening of hemicellulose cross-linking to microfibrils and the associated expansion process (Carpita and Gibeaut, 1993). Many enzymes have been shown to be associated with hemicellulose. The enzyme xyloglucan endotransglycosylase is an important entity that is thought to be responsible for the incorporation of XG into the wall matrix (Darley, et al., 2001). It is a key mediator regulating cell wall expansion. This matrix polysaccharide is produced in the Golgi apparatus and become integrated into the existing wall by enzymes and probably by exocytosis and/or unknown binding mechanisms.

Primary cell-wall enlargement also involves wall stress relaxation, which allows the cells to take up water and physically enlarge, a process involving many proteins. For example expansins are nonenzymatic wall proteins and induce wall stress relaxation and extension (Cosgrove, 1996). They mediate 'acid-induced growth' by disrupting the non-covalent linkages involved with microfibrils of the cell wall (Cosgrove, 2005). As cell expansion approaches

cessation, secondary wall deposition takes place inside the primary cell wall in certain cells (Figure 1.1).

1.1.2 Secondary cell wall

In certain plant cell types, a thick secondary cell wall inside the primary wall is laid down once expansion has ceased (Figure 1.2). In contrast to the primary wall, the secondary cell wall is a strong multilayered structure whose presence inhibits any further cell elongation. The main function of the secondary cell wall in plants is to provide strength and facilitate water and nutrient uptake. It forms a strong hydrophobic layer inside the water conducting tissues (xylem) and protects them from desiccation. Very tight spatial and temporal regulation of secondary wall deposition has evolved in plants, equiping various cell types with secondary walls that differ in form and composition. These specialized cells are organized in tissues to collectively form a functional plant (Raven et, al. 1999). Certain cells like sclereids and fibers have evenly thickened secondary walls, except for certain areas like pits where cell wall thickening are absent. The secondary wall thickening in tracheary elements, on the other hand, has distinct patterns of deposition, namely, annular (cell wall is laid down in rings) and/or helical (cell wall deposited in helical pattern). These localized secondary structures are common in cells that have not yet finished elongating; for example, those in protoxylem. In addition, tracheary elements show reticulated, scleriform, and pitted wall thickening patterns that permit little further elongation. This occurs in tissues such as metaxylem and secondary xylem, which have already completed most or all of their elongation. This again indicates a very tight regulation of secondary cell wall both in space and time.

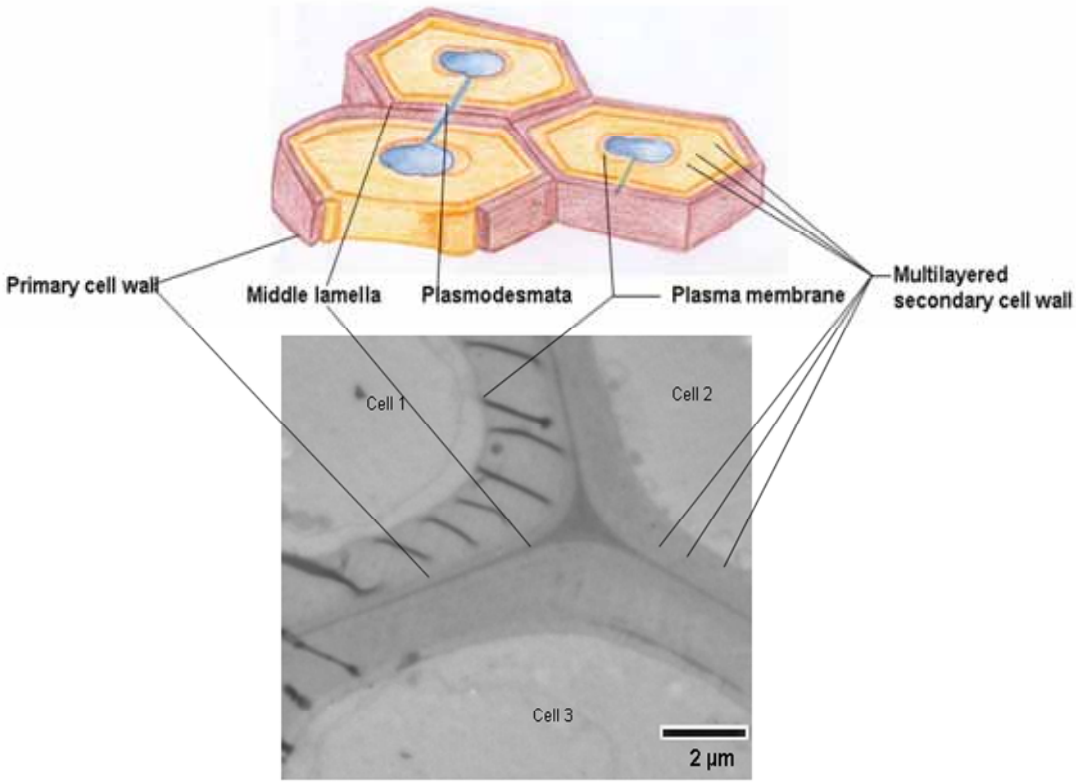


Figure 1.2. Secondary cell wall. Diagram depicting three dimensional structure of a plant cell and position of the secondary cell wall (top) and TEM (below) showing secondary cell wall as a thick, multilayered cell wall which is deposited inside the primary cell wall. Scale= 2µm (Drawing by V. Bhargava, used with permission).

Secondary cell wall: Cellulose, hemicellulose and lignin biosynthesis

Cellulose is often the most abundant component of plant secondary cell walls and can make up a large proportion of the dry weight of plants. In addition to cellulose, hemicellulose and lignin are also important components of the secondary cell wall. Cellulose is a polymer of unbranched β-1,4-linked glucan chains and is the major component of most plant cell walls, including secondary cell walls. Rosettes, the complexes on the plasma membrane that are also called cellulose synthase complexes, are responsible for cellulose synthesis. These complexes consist of subunits encoded by *CESA* (cellulose synthase) genes (Somerville, 2006). In Arabidopsis, CESA4, CESA7 and CESA8 are generally thought to be involved in cellulose biosynthesis in secondary cell walls (Taylor, et al., 2004). However, CESA9, which is normally associated with

primary cell wall formation, was also recently shown to have a specific role in secondary cell wall formation in the Arabidopsis seed coat (Stork, et al., 2010). There is also evidence that cortical microtubules play roles in the deposition of the secondary walls. This is evident in differentiation of tracheary elements (Oda, et al., 2005). In Arabidopsis, co-localization of cortical microtubules with CesA4, 7 and 8, the secondary cell wall-associated CESA proteins, was observed in xylary vessels (Gardiner, et al., 2003). This indicated that microtubules may direct the deposition of the cellulose synthase complexes in the plasma membrane indicating that microtubules are integral part of cellulose synthesis machinery during the secondary cell wall synthesis (Gardiner, et al., 2003; Wightman and Turner, 2008). It is also speculated that microtubules may be required to establish the orientation of cellulose microfibril deposition (Roberts, et al., 2004; Sugimoto, et al., 2003; Wasteneys, 2004).

In dicotyledonous plants, the main hemicellulosic polysaccharides found in secondary walls are xylans. In this polymer, the backbone is composed of xylosyl residues along with glucuronic acid residues. Many glucuronyltransferases involved in the biosynthesis of glucuronoxylan during secondary wall formation have been characterized earlier (Persson, et al., 2007; Brown, et al., 2009).

Lignin is derived through the phenylpropanoid pathway and is hydrophopic in nature. It is a polymer of monolignols that polymerize to form a three-dimensional matrix. In the secondary cell wall, it impregnates the cellulose and hemicellulose network and not only is responsible for providing mechanical strength but also makes secondary cell wall water impermeable. The phenylpropanoid pathway is responsible for monolignol biosynthesis in the cytoplasm and these constituents of lignin are transported to the cell wall via an unknown mechanism. Lignin biosynthesis and the phenylpropanoid pathway have been well-studied and characterized in many plants, especially in Arabidopsis. Forward and reverse genetics and biochemical analysis have contributed to the characterization of most of the enzymes involved in this pathway. (Humphreys and Chapple, 2002; Boerjan, et al., 2003). The first step in the general phenylpropanoid pathway is the enzymatic conversion of phenylalanine to cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL). Further, the hydroxylation of cinnamic acid to coumaric acid by P450 mono-oxygenase, C4H, and the formation of coenzyme A thioesters of hydroxycinnamoyl intermediates by a ligase (4CL), provide precursors for branch pathways leading to the production of a number of phenolics, including lignin, soluble esters, coumarins, and flavonoids

(Hahlbrock and Scheel, 1987). In addition to phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), (Lee, et al., 1997) and 4-(hydroxy) cinnamoyl CoA ligase (4CL), the other enzymes involved in the biosynthesis of monolignols are hydroxycinnamoyl CoA;shikimate hydroxycinnamoyl transferase (HCT), p-coumaroyl shikimate 3'-hydroxylase (C3H), caffeoyl CoA O-methyltransferase (CCoAOMT), (hydroxyl) cinnamoyl CoA reductase (CCR), ferulic acid 5 hydroxylase (F5H), caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT) and (hydroxy) cinnamyl alcohol dehydrogenase (CAD). In the end, three kinds of monolignol subunits, hydroxyphenyl (H), guaiacyl (G) and syringyl (S) subunits are derived from three monolignols: coumaryl alcohol, coniferyl alcohol and sinapyl alcohol respectively. Biosynthesis of S units requires the ferulate 5-hydroxylase (F5H) enzyme (Humphreys and Chapple, 2002).

Once monolignols are produced in cells and exported to secondary walls, their polymerization to form a network of ester, ether, and carbon-carbon linkages typical of functional lignin is thought to be catalyzed by peroxidases (PER) and/or laccases (LAC) (Boerjan, et al., 2003; Raes, et al., 2003), but the exact mode of action of these enzymes in the lignin polymer formation *in planta* is not known. G and S are the major units found in the lignin of angiosperms but gymnosperm lignin is devoid of S units. In most of the plants, H and S subunits are deposited earlier in lignification process (Boerjan, et al., 2003) .

Secondary cell walls as a carbon sink

Allocation of carbon to different metabolic pathways is an important aspect of growth and development in plants (Bloom, et al., 1985; Smith and Stitt, 2007), including the formation of secondary cell walls. As soon as cell expansion and growth have stopped, the deposition of secondary cell wall constituents like lignin, cellulose and hemicellulose is initiated in certain cell types (Somerville, 2006; Lerouxel, et al., 2006; Zhong and Ye, 2007; Ye, et al., 2002). The building blocks for cellulose and hemicellulose biosynthesis are provided by the major carbon metabolic pathways in the cell, such as glycolysis and the pentose phosphate pathway (Figure 1.3).

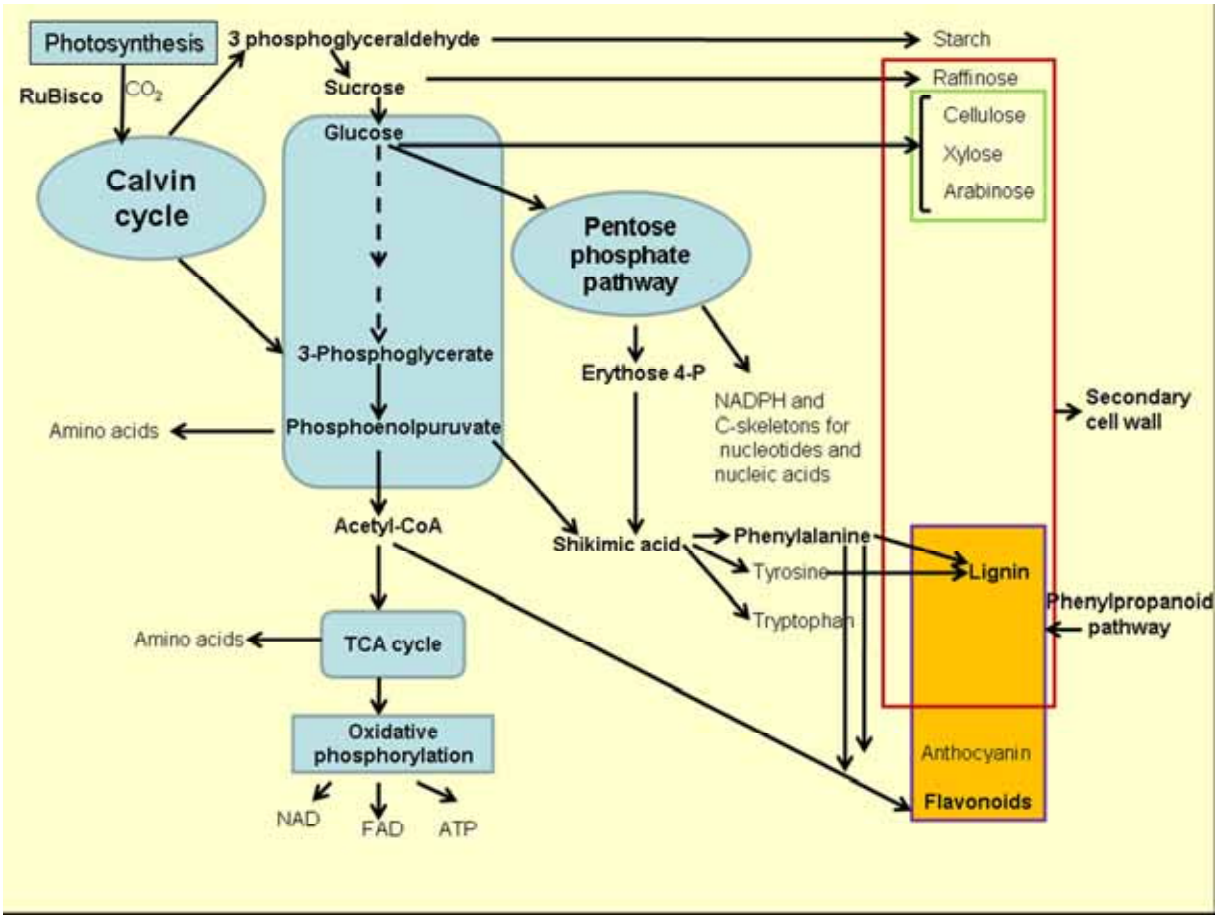


Figure 1.3 Carbon metabolism in a plant cell. This carbon sink acts as a donor for carbon skeleton in secondary cell wall. Carbon skeletons for secondary cell wall (Cellulose, hemicellulose and lignin) are provided by different metabolic pathways in the cell.

The phenylpropanoid pathway also draws on these central carbon pathways through their provision of carbon skeletons to the shikimate pathway, which generates the aromatic amino acids, including L-phenylalanine. In cells that are heavily committed to lignin deposition in the secondary cell wall, this aspect of cell wall metabolism represents another important sink for photosynthetic derived carbon in plants (Hahlbrock and Scheel, 1987). In order to generate a sufficient supply of carbon skeletons for phenylpropanoid biosynthesis, the shikimate pathway, that is responsible for biosynthesis of aromatic amino acid, must be regulated in a fashion that coordinates the metabolic demand for phenylalanine as the primary precursor of phenylpropanoid pathway with the metabolic demand for all three aromatic amino acids as precursors for protein formation

There is some evidence for the coordination of shikimate and phenylpropanoid metabolism in the context of lignin formation in Arabidopsis (Lee, et al., 1997). 4-(hydroxy)

cinnamoyl CoA ligase (4CL) is an important enzyme in the general phenylpropanoid pathway. Earlier work by Lee et al. (1997) showed that partial blocking of 4CL activity had opposite affects on the accumulation of G and S lignin monomers and indicated reduced carbon flux through the phenylpropanoid pathway. This may imply the role of an uncharacterized pathway for the biosynthesis of S lignin, independent of 4CL activity or a role of phenylpropanoid-associated pathways like shikimate pathway.

The regulation of carbon allocation and its commitment to different pathways is thought to be determined by many structural and regulatory genes. However, the proteins and genes that control and regulate general carbon partitioning, allocation and distribution in plants have not been identified or fully characterized (Smith and Stitt, 2007). Specifically for secondary cell wall carbon redistribution in a cell, very little information is available in the literature. Components of the carbon signaling and sensing system(s), metabolism and cell wall or matrix, are all hypothesized to be parts of an integrated system that is connected with other assimilatory and metabolic pathways (Coruzzi and Bush, 2001; Coruzzi and Zhou, 2001). For example, the ability to sense carbon and nitrogen metabolites enables plants to regulate metabolism. Sugar-sensing mechanisms in plants allow them to down regulate photosynthesis when C-skeletons are in excess. Similarly, the C:N-sensing mechanism together enables plants to activate genes involved in N-assimilation when C skeletons are abundant and internal levels of organic-N are low, suggesting that two different but interdependent assimilatory pathways have to be tightly regulated and coordinated.

The relationship between closely related biosynthetic pathways (e.g. anthocyanin/ flavonoids relative to the lignin pathway) in maintaining carbon homeostasis during the process of secondary cell wall formation has not been examined. Maintenance of an appropriate balance of carbon allocation for the various building blocks of the secondary cell wall would be predicted to be very important in a plant cell as it undergoing changes after growth has stopped. Since transcription factors have the ability to integrate developmental and environmental cues, and to co-ordinate activity in different metabolically associated pathways, they are important candidates for this kind of regulation in Arabidopsis.

1.2 TRANSCRIPTION FACTORS AND THEIR ROLE IN REGULATION OF SECONDARY CELL WALL FORMATION

The interaction of general and tissue-specific transcription factors with each other and/or with specific regions of genomic DNA is thought to regulate the complex patterns of gene expression observed both spatially and temporally in development of all eukaryotic organisms (Izawa, et al., 1993; Menkens, et al., 1995). A number of different components are required for the process of transcription, including chromatin remodeling factors, the pre-initiation complex and the RNA polymerase II complex. Transcription factors (TF) that control the promoter activity by recognizing DNA in a sequence-specific manner and then regulate the frequency of initiation of transcription are also required in this process. Activator transcription factors may activate RNA polymerase and enhance gene expression. Conversely, repressor transcription factors inhibit transcription by blocking the attachment of activator proteins. Some transcription factors can act as either an activator or repressor, depending on the context (Ikeda, et al., 2009).

1.2.1 Transcription factor families in plants: Role of MYB and KNOX family members in gene regulation in plants

Transcription factors are generally modular in nature and usually contain a DNA-binding domain that recognizes DNA in a sequence-specific manner, an activator or a repressor domain and a protein-protein interaction domain. Based on the presence of conserved DNA binding domains, plant transcription factors have been classified into different families (Pabo and Sauer, 1992; Qu and Zhu, 2006; Riechmann, et al., 2000). In Arabidopsis these include Myb-like (MYB) proteins, basic region-leucine zipper (bZIP) proteins, MADS domain proteins, helix-loop-helix proteins (bHLH), zinc-finger proteins, and homeo-box proteins (Meshi and Iwabuchi, 1995; Schwechheimer, et al., 1998).

The basic helix-loop-helix zipper (bHLH) genes constitute one of the largest families of transcription factors in Arabidopsis, and have been shown to regulate broad-spectrum of functions (Heim, et al., 2003; Rosinski and Atchley, 1999). Two important regions of bHLH transcription factors are N-terminal transactivation domain and a C-terminal basic helix-loop-helix (bHLH) leucine zipper motif.

MADS domain transcription factors with a conserved DNA binding domain, known as the MADS domain, constitute a large family of regulatory factors (Pellegrini, et al., 1995; Theissen, et al., 1996). These transcription factors have been shown to play important roles in regulating many different developmental processes and are found in all eukaryotic organisms. However, in plants, they form a particularly diverse group. MADS proteins in plants not only have DNA binding domain but also have a protein–protein interaction domain (Riechmann and Meyerowitz, 1997). This family includes flower meristem identity regulators APETALA1 (AP1), silique tissue identity regulators AGL8, AGL1, and AGL5 and the floral organ identity regulators AGAMOUS (AG), APETALA3 (AP3) and PISTILLATA.

The transcription factors AP2 (APETALA2) and EREBPs (ethylene-responsive element binding proteins) belong to a plant-specific family of AP2-EREBP transcription factors that contain the AP2 DNA-binding domain and are part of a large multigene family. They have been shown to be involved in a broad spectrum of regulation in plants, including the signaling associated with various types of biotic and environmental stress (Riechmann and Meyerowitz, 1998).

Zinc finger proteins constitute a large family of TFs in the genomes of higher and lower eukaryotes. They show a wide range of functions, including DNA binding, RNA binding or involvement in protein-protein interactions. Several finger-like protrusions interact with metals like zinc to form the conserved domain in these TFs. These proteins also contain specialized interaction domains. Some of these domains have been shown to bind DNA, as observed in Dof and WRKY proteins. Others bind to RNA as in the case of the SERRATE, a microRNA processing factor (Yanagisawa and Schmidt, 1999; Yanagisawa, et al., 2004; Yang, et al., 2006). Several TF's from this family have been functionally characterized in Arabidopsis. They are involved in a multiple physiological processes including floral differentiation, leaf and lateral shoot initiation and regulation of stress responses (Takatsuji, 1999). In the following section, I will discuss the plant-specific MYB and homeo-domain families and their regulatory roles in plant development in more detail.

MYB

The MYB family of transcription factors is a set of functionally diverse transcriptional factors that are found in both plants and animals. In plants, it is one of the largest families of transcription factors identified to date and is thought to contain approximately 125 genes in Arabidopsis (Riechmann, et al., 2000; Stracke, et al., 2001). The large number of *MYB* genes present in plants is dramatically different from the relatively small number of *MYB* genes reported to be encoded in other eukaryotic genomes (Stracke, et al., 2001; Stober-Grasser, et al., 1992). These genes encode proteins with a DNA-binding domain that consists of one to four imperfect tandem repeats (R1, R2, R3, or R4), which collectively make up the MYB domain (Rosinski and Atchley, 1998; Yanhui, et al., 2006). These TFs also have a characteristic central tryptophan cluster in the three-dimensional helix-turn-helix fold because of the presence of regularly spaced tryptophan residues in MYB repeats (Kanei-Ishii, et al., 1990).

The majority of plant *MYB* genes encode proteins with only two repeats which are most similar to the vertebrate R2 and R3 MYB repeats (R2R3) (Stracke, et al., 2001; Rosinski and Atchley, 1998; Braun and Grotewold, 1999). However, a small family of R1R2R3 MYB domain proteins is also found in plants. Therefore, these plant homologues (R2R3) are thought to have evolved from an ancestral *R1R2R3-MYB* gene by loss of sequences encoding the R1 repeat and following expansion of this gene family (Rosinski and Atchley, 1998). Plant R2R3 *MYB* genes have evolved to develop diverse functional roles in the life of plants (Dubos, et al., 2010). In recent years, using both genetic and molecular analyses, the functions of MYB proteins have been studied in numerous plant species such as Arabidopsis, rice (*Oryza sativa*), maize (*Zea maize*), snapdragon (*Antirrhinum majus*), petunia (*Petunia hybrida*), poplar (*Populus tremuloides*), apple (*Malus domestica*) and grapevine (*Vitis vinifera* L.) (Yanhui, et al., 2006; Wilkins, et al., 2009; Zhang, et al., 2010; Feng, et al., 2010; Tamagnone, et al., 1998; Palapol, et al., 2009; Ban, et al., 2007; Matus, et al., 2008; Quattrocchio, et al., 2006).

Numerous R2R3-MYB proteins have been found to be involved in the control of many plant-specific processes including metabolism both primary and secondary, cell differentiation, and responses to both biotic and abiotic stresses and have been well studied in Arabidopsis (Table 1.1) (Dubos, et al., 2010).

Table 1.1. Examples of R2R3 MYB transcription factors involved in the control of plant-specific processes.		
Development, cell fate and identity		
Name	**Function**	**Reference**
MYB0/GL1	formation of trichomes	(Oppenheimer, et al., 1991)
MYB66/WER	root hair formation	(Schiefelbein, 2000)
MYB106/MIXTA	cell shape	(Noda, et al., 1994)
MYB103	anther development	(Zhang, et al., 2007)
MYB124/FLP	stomatal development	(Lai, et al., 2005)
MYB61	stomatal aperture	(Liang, et al., 2005)
Name	**Function**	**Reference**
Metabolism		
MYB75/PAP1 MYB113 MYB114 MYB90/PAP2	anthocyanin pigments	(Gonzalez, et al., 2008)
MYB12/PFG1 and MYB11/PFG3	flavonols	(Mehrtens, et al., 2005)
	phenolics	(Dubos, et al., 2010)
MYB28/HAG1/PMG1, MYB29/HAG3/PMG2, MYB76/HAG2, MYB34/ATR1, MYB51/HIG1, MYB122	glucosinolate biosynthesis	(Gigolashvili, et al., 2007; Gigolashvili, et al., 2008).
MYB123/TT2	proanthocyanidins	(Lepiniec, et al., 2006)
MYB5, MYB123	tannins	(Li, et al., 2009b)
MYB77 MYB62	hormone biosynthesis/signaling	(Shin, et al., 2007; Devaiah, et al., 2009)
Name	**Function**	**Reference**
Biotic and abiotic stress		
MYB1	response to viral attack	(Yang and Klessig, 1996; Urao, et al., 1993)
MYB2	response to drought	(Urao, et al., 1993)
MYB4	response to UV light	(Jin, et al., 2000)

In addition to the above shown functions (Table 1.1), MYB transcription factors have been shown to be involved in regulating vascular differentiation and phenylpropanoid gene expression (Jin, et al., 2000; Bonke, et al., 2003; Borevitz, et al., 2000). The phenylpropanoid pathway is an important contributor to secondary cell wall constituents including lignin (Figure 1.3). The other branches of phenylpropanoid pathway are also regulated by these TFs. For example, in Arabidopsis several R2R3-MYBs (MYB11, MYB12 and MYB111) are involved in the regulation of flavonoid biosynthesis (Stracke, et al., 2007), proanthocyanidins (PAs) in the seed coat (MYB123/TT2) (Lepiniec, et al., 2006), anthocyanin biosynthesis (MYB75/PAP1,

MYB90/PAP2, MYB113 and MYB114) (Gonzalez, et al., 2008) and tannin biosynthesis (MYB5, MYB123) (Li, et al., 2009b). These regulators can act either as transcriptional activators or repressors in regulating the biosynthesis and accumulation of these phenylpropanoid compounds. Repressors, including MYB3, control flavonoid biosynthesis (Dubos, et al., 2008), while MYB4 controls sinapate ester biosynthesis in a UV-dependent manner (Jin, et al., 2000), and MYB32 regulates pollen wall composition (Preston, et al., 2004).

Regulation of secondary cell wall formation, either broad regulation or regulation of specific constituent (cellulose, hemicellulose and lignin) is also controlled by MYBs in addition to their roles in phenylpropanoid pathway regulation. MYB58, MYB63 and MYB85 are known to activate lignin biosynthesis in fibers and/or vessels (Zhou, et al., 2009; Zhong, et al., 2008) and, MYB68 has been shown to negatively regulate lignin deposition in roots (Feng, et al., 2004). While MYB46 positively regulates cellulose, xylan and lignin biosynthesis in fibers and vessels (Zhong, et al., 2007a), MYB26 controls secondary wall deposition in anthers (Yang, et al., 2007). Similarly, the broader regulatory role in stems is shown by MYB52, MYB54 and MYB69 which are thought to regulate lignin, xylan and cellulose biosynthesis (Zhong, et al., 2008). MYB61 plays a broader and pleiotropic role, influencing not only lignin deposition (Newman, et al., 2004). but also mucilage production (Penfield, et al., 2001) and stomatal aperture (Liang, et al., 2005). Therefore, MYB transcription factors can have multiple roles whose impacts are regulated in turn over space and time.

One of the MYB transcription factors in Arabidopsis that has been shown to regulate multiple physiological functions is MYB75. MYB75, also known as PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT 1), is an R2-R3 family member (Figure 1.4) and has been characterized as positively regulating the anthocyanin and proanthocyanidin branches of the phenylpropanoid pathway (Gonzalez, et al., 2008; Borevitz, et al., 2000; Pourtau, et al., 2006; Tohge, et al., 2005b; Tohge, et al., 2005a; Matsui, et al., 2004), However, it has also been implicated in radical scavenging (Tohge, et al., 2005a), sucrose signaling (Teng, et al., 2005), nitrogen starvation responses (Lea, et al., 2007), phosphorus-deprivation responses (Morcuende, et al., 2007) and senescence (Bernhardt, et al., 2003). Previous work by Borevitz et al. (2000) using a *MYB75* over-expression mutant revealed changes in hydroxycinnamic acid content in the tissues of this mutant, thus indicating a role for PAP1 in regulating not only anthocyanin biosynthesis but also other branches of the phenylpropanoid pathway in Arabidopsis.

Figure 1.4 Cartoon indicating structure of an R2-R3 MYB transcription factor

Genetic redundancy in plants is an important concern for functional characterization of large gene families such as the MYB transcription factors, since loss-of-function mutant analysis often does not result in a phenotype. For example, anthocyanin regulators, *MYB75*, *MYB90*, *MYB113* and *MYB114* have high sequence similarity that results in functional redundancy (Gonzalez, et al., 2008). Therefore, to understand the complete anthocyanin pathway regulation by these MYBs, multiple mutants might be required (Gonzalez, et al., 2008). The structural similarity of certain MYB TFs is also apparently responsible for functional redundancy. For example, MYB66/WER and MYB0/GL1 are structurally closely related proteins and also show functional similarities (Lee and Schiefelbein, 2001).

Many studies have looked at different aspects of control regulated by these MYB transcription factors. Numerous target genes were identified and many mechanisms have been proposed. Roles of miRNA, other transcription factors, feed back metabolic loops and other transcription factors regulating the MYB TFs have been suggested. In addition, the regulatory role of these MYBs on the target genes have suggested that MYB family members may bind to AC elements to regulate transcription from promoters containing these elements (Sugimoto, et al., 2000). This has been evident from a detailed analysis of genes encoding enzymes implicated in lignin biosynthesis (Rogers, et al., 2005). In addition to specificity provided by MYB-DNA interactions, protein-protein interactions (Zimmermann, et al., 2004) account for the multiplicity of function and formation of different complexes in different combinations which are the key for the functional diversity.

Homeodomain

Homeodomain transcription factors also known as homeotic factors, have been implicated to be involved in a number of developmental processes including the control of pattern formation in insects and vertebrate embryos, and the specification of cell differentiation in many tissues in

animals (Mukherjee, et al., 2009; Ingham, 1998; McGinnis and Krumlauf, 1992). Plant homeodomain genes have been identified in a number of plant species including Arabidopsis, and have a highly conserved 60-amino acid motif DNA-binding domain called the homeodomain (Gehring, 1987). This domain folds into a three-helix tertiary structure. Homeodomain proteins have been divided into two classes based on their conserved amino acid sequences, namely, typical, with a 60 amino acid homeodomain and TALE (Three-amino-acid-loop extension) proteins, which possess an extra three amino acids (Pro-Tyr-Pro) found between helices I and II (Bertolino, et al., 1995; Mukherjee and Burglin, 2007; Burglin, 1997).

In plants, five homeodomain TF families are present while only two TALE gene family classes exist. The two families of TALE family include KNOTTED1-like homeobox (KNOX) and BEL1-like homeobox (BLH)(Mukherjee and Burglin, 2007; Hake, et al., 2004). TALE homeodomain proteins also play key roles in developmental programs in plants similar to seen in animals and fungi (Mukherjee and Burglin, 2007; Burglin, 1997). Plant KNOX proteins have an ELK domain and a KNOX (MEINOX) domain (Burglin, 1997) and are present in both higher and lower plants (Hake, et al., 2004; Reiser, et al., 2000; Lincoln, et al., 1994; Sakakibara, et al., 2008). This family of TALE protein has eight characterized members that are divided in to two sub-classes based on sequence similarity and expression patterns (Kerstetter, et al., 1994). *STM, BREVIPEDICELLUS (BP)/KNAT1, KNAT2* and *KNAT6* belong to class I and are expressed in and regulate shoot apical meristem (SAM) (Lincoln, et al., 1994; Long, et al., 1996; Douglas, et al., 2002; Belles-Boix, et al., 2006), while *KNAT3, KNAT4, KNAT5* and *KNAT7* belong to class II (Hake, et al., 2004) and their functions are less well understood.

One member of the class II KNOX family, *KNAT7* appears to have a role in the regulation of secondary cell wall formation. *KNAT7* has been shown as a potential regulator of secondary wall biosynthesis based on its co-expression with secondary cell wall biosynthetic enzymes (Persson, et al., 2005; Brown, et al., 2005; Ehlting, et al., 2005). Later it was shown that a *knat7* loss-of-function mutant possesses an *irregular xylem* (*irx*) phenotype (Brown, et al., 2005). In addition to its *irx* phenotype, *knat7* mutants have increased cell wall thickness in their interfascicular fibers (Li, 2009). Furthermore, KNAT7 acts as a transcriptional repressor when transiently expressed in Arabidopsis protoplasts (Li, 2009) consistent with its proposed role as a negative regulator of secondary cell wall formation in Arabidopsis interfascicular fibers (Li,

2009). In addition to its DNA binding function, KNAT7 has also been found to display protein-protein interactions with other transcription factors (Li, 2009; Hackbusch, et al., 2005).

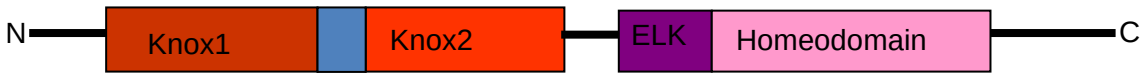


Figure 1.5 Cartoon indicating structure of KNAT7, a homeodomain transcription factor.

1.2.2 Transcription factors, protein-protein interactions and protein complex formation in plants

Expression of a given gene is often regulated by more than one transcription factor. This complex regulation presumably allows different transcription factors to respond to different stimuli and form different complexes that effectively integrate incoming signals to regulate different biological processes and responses. The pattern of interactions may have synergistic effects on the binding of specific TFs to a given promoter and hence allow the fine-tuning of the regulation of its expression. It should be noted that these complexes may either activate or repress the expression of their target gene. Hence, both the diversity as well as the fine-tuning of the regulation by the transcription factors is thought to be provided, in part, by the interacting protein partners in such transcriptional complexes (Uhrig, 2006). In addition, this combinatorial nature of transcriptional control in eukaryotic cells allows for the generation of extensive regulatory diversity by a relatively limited number of factors. There are many reported direct interactions between different plant TFs (Singh, 1998). For example, in plants, the cooperation between TFs was earlier demonstrated to be important for the expression of flavonoid biosynthesis genes (Goff, et al., 1992), ABA regulated gene expression (Abe, et al., 2003), for trichome development (Payne, et al., 2000), for phenylpropanoid pathways (Gonzalez, et al., 2008; Fornale, et al., 2006) as well as many other developmental processes (Penfield, et al., 2001; Zhang, et al., 2003). Protein interactions of MYBs and homeodomain transcription factors are discussed below.

MYB transcription factors, complex formation and protein-protein interactions

Regulatory activity of MYB transcription factors is dominated by protein–protein interactions. R2R3 or R1R2R3 MYB proteins bind DNA *in vitro* as monomers, (Ogata, et al., 1995) while heterodimerization and homodimerization is shown by MYB proteins with one MYB repeat (Lu, et al., 2009). Because of these interactions, these proteins recognize DNA with high affinity and specificity. The transcriptional activity of some R2R3-MYB factors has been shown to be dependent *in vivo* on protein–protein interactions as seen between R2R3-MYB factors and basic helix–loop–helix (bHLH) proteins. MYB R3 repeat has been shown to be important in providing the specificity of the interaction between MYB and bHLH proteins (Zimmermann, et al., 2004; Grotewold, et al., 2000). Interactions with other proteins can also modulate MYB activities in Arabidopsis, as exemplified by the interactions of MYB30 (Yang, et al., 2009), MYB77 and MYB18 with partner proteins (Shin, et al., 2007; Yang, et al., 2009; Li, et al., 2009a). Hence, protein-protein interactions in MYBs are important for providing specificity within their broader biological functionality.

Homeodomain transcription factors, complex formation and protein-protein interactions

Protein–protein interactions are thought to be crucial for homeodomain protein function. It has been shown earlier that the MEINOX (KNOX1 and KNOX2) domain is important for interaction BELL proteins (Bellaoui, et al., 2001; Kumar, 2006; Muller, et al., 2001; Smith, et al., 2002; Bhatt, et al., 2004). Interactions of KNOX and BLH proteins have been described earlier (Muller, et al., 2001; Viola and Gonzalez, 2006) and their putative complexes are thought to regulate plant development. The ELK domain is hypothesized to play role in nuclear localization, protein-protein interaction and regulating gene activation (Nagasaki, et al., 2001; Meisel and Lam, 1996). Members of the KNOX family also interact with members of the Arabidopsis OVATE protein family (OFP) (Hackbusch, et al., 2005) as shown by KNAT7, that has been shown earlier to bind to OFP4 proteins (Li, 2009; Hackbusch, et al., 2005). Therefore, protein-protein interactions are also important features of homeodomain TF function and such interactions could reflect specific roles for these complexes in the regulation of plant development, including secondary cell wall formation in Arabidopsis.

1.2.3 Transcription factors and regulation of secondary cell wall formation

Transcription factors are known to be important regulators of most of the structural and functional phenomena in plant development including secondary cell wall formation. Molecular and genetic studies in Arabidopsis have elucidated important insights in the transcriptional regulation mechanisms of secondary wall formation. However, very little information is available regarding the spatial and temporal pattern of TF regulation of secondary cell wall biosynthesis in plants. The existing studies have resulted in developing a model for a transcriptional network that with a cascade of transcription factors that may be involved in the regulation of secondary wall formation in different cell types (Zhong and Ye, 2007). A number of these transcription factors in Arabidopsis have been characterized by reverse genetics and many more have been postulated to play a role in this regulation based on co-expression analyses (Ehlting, et al., 2005). In addition to those TFs direct regulating the secondary cell wall biosynthetic machinery, regulators involved in controlling other biosynthetic pathways that feed into the secondary cell wall-associated pathways (cellulose, hemicellulose, lignin etc.) are also likely to contribute to secondary cell wall regulation by maintaining the homeostasis of carbon supply.

Aside from direct TF control of the secondary cell wall formation process, many transcription factor classes have been related with the broader regulation of vascular system formation and development. Examples of these include the KANADI transcription factor, an auxin response factor (a class III homeodomain-leucine zipper), involved in determination of xylem-phloem identity (Ilegems, et al., 2010) and some NAM/ATAF/CUC (NAC) family proteins, involved in differentiation of various cell types into cells with thickened secondary walls (Yamaguchi, et al., 2008; Yamaguchi, et al., 2010). Some of these TFs are also may result in forming transcriptional networks (Demura and Fukuda, 2007; Du and Groover, 2010).

Some of the NAC domain transcription factors like VASCULAR-RELATED NAC DOMAIN6 (VND6) and VND7, SECONDARY WALL–ASSOCIATED NAC DOMAIN PROTEIN1 (SND1), NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1), NST2 function at the top of secondary cell wall regulatory network in the proposed model (Figure 1.6) to activate the entire secondary wall biosynthesis (Yamaguchi, et al., 2008; Kubo, et al., 2005; Mitsuda, et al., 2007; Mitsuda and Ohme-Takagi, 2009; Zhong, et al., 2007b; Zhong, et al., 2006; Mitsuda, et al., 2005; Grant, et al., 2010). Both SND1 and NST1 a have a role in the development of secondary cell wall in fibers (Mitsuda, et al., 2007; Zhong, et al.,

2007b; Zhong, et al., 2006). Another member of NAC, XND1, has been shown to negatively regulate lignocellulose biosynthesis in xylem cells of Arabidopsis (Zhao, et al., 2008). However, some of these TFs can also have distinct roles in different tissues/cells. For example, NST1 and NST3 are both regulators of secondary wall formation in the anthers (Mitsuda, et al., 2005), but NST1 along with NST2, is also involved in regulating secondary wall formation in woody tissues of Arabidopsis (Mitsuda, et al., 2007). Similarly, VND6 and VND7 are both involved in regulating secondary wall formation in vessels of Arabidopsis (Kubo, et al., 2005) but VND7 is specifically involved in regulating the differentiation of root protoxylem vessels (Yamaguchi, et al., 2008; Yamaguchi, et al., 2010). These NACs TFs can activate transcription factors that may lie downstream, and may activate the secondary wall biosynthetic genes directly or indirectly. The direct targets of SND1 and its close homologs, including NST1, NST2, VND6 and VND7 have been identified and include MYB46, SND3, MYB103 and KNAT7(Zhou, et al., 2009; Zhong, et al., 2007a; McCarthy, et al., 2009). MYB46 and MYB83 can also regulate the entire secondary wall biosynthetic network (Ko, et al., 2009; McCarthy, et al., 2009).

Many transcription factors have also been specifically assigned to secondary cell wall regulation in other tissues like seed coat in Arabidopsis. For example, APETALA2, TTG1 (WD), TT8 (bHLH), EGL3 (bHLH), TTG2 (WRKY44) GL2 (Homeodomain)(Haughn and Chaudhury, 2005; Gonzalez, et al., 2009), KNAT7 (Li, 2009), MYB5 (Li, et al., 2009b; Gonzalez, et al., 2009) and MYB61 (Penfield, et al., 2001) have been shown to affect mucilage and/or epidermal development in the seed coat, along with the secondary cell wall regulation (Figure 1.6). However, the distinction between the seed coat mucilage biosynthesis transcription factors and secondary cell wall formation regulators is presently not clear.

MYBs and regulation of secondary cell wall formation

Members of the MYB family also have conserved regulatory roles in secondary cell wall formation (Figure 1.6). Many members of the R2R3 MYB protein families have been anticipated as transcriptional regulators of the phenylpropanoid biosynthesis pathway and secondary cell wall formation (Bomal, et al., 2008; Fornale, et al., 2006; Goicoechea, et al., 2005; Zhou, et al., 2009; Patzlaff, et al., 2003a). In addition to MYB46 and MYB83 mentioned above, a number of other Arabidopsis MYBs have been characterized for their involvement in regulation of secondary cell wall formation. Arabidopsis MYB26 has been shown to regulate secondary

thickening in the anthers and is involved in their dehiscence (Yang, et al., 2007). MYB58 and MYB63 (Zhou, et al., 2009) have been shown to be direct regulators of the lignin biosynthetic pathway in Arabidopsis and more specifically in its stems. MYB61 (Newman, et al., 2004) plays a broader role in carbon sensing and is involved in other functions in addition to regulate some aspects of secondary cell wall in Arabidopsis (Liang et al., 2005).

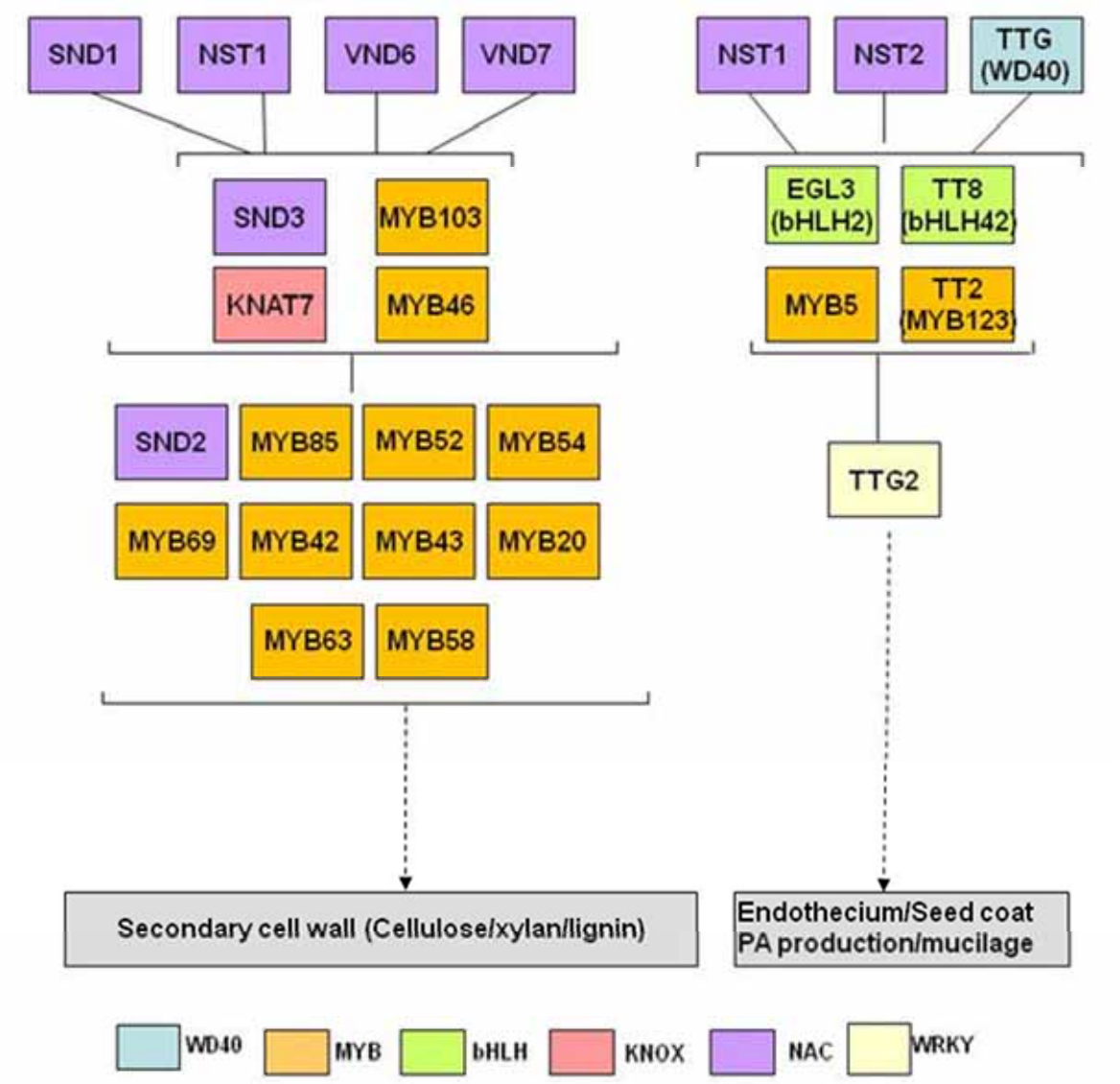


Figure 1.6 Model of the proposed transcriptional network regulating secondary cell wall (Adapted from McCarthy et al., 2009).

The regulatory role of these MYB transcription factors in secondary cell wall formation has also been characterized in other species in addition to Arabidopsis. Secondary xylem development in wood has been shown to be regulated by *MYB* genes in pine (*Pinus taeda*; *Pt MYB1* and *Pt MYB4*) and eucalyptus (*Eucalyptus grandis*; *Eg MYB2*)(Goicoechea, et al., 2005; Patzlaff, et al., 2003a). These *MYB* genes from pine and eucalyptus are proposed to be regulators of lignin biosynthesis during secondary growth involving secondary cell wall biosynthesis. These TFs are homologs of Arabidopsis MYB46 (Goicoechea, et al., 2005), which is a master regulator of the biosynthesis of cellulose, xylan, and lignin (Zhong, et al., 2007a; Ko, et al., 2009; McCarthy, et al., 2009). Similarly, PttMYB21 from hybrid aspen (Karpinska, et al., 2004) and PtrMYB3 and PtrMYB20 from poplar (McCarthy, et al., 2010) were shown to be expressed and regulated during secondary vascular development,

In recent years, a large amount of information has been generated in the field of cell wall study and has enhanced our understanding of the regulatory network involving TFs and regulating secondary wall biosynthesis. However, identifying all the players and their roles in the transcriptional network regulating secondary cell wall is a challenge to achieve in future studies.

1.4 RESEARCH OBJECTIVES

The main objectives of my thesis were motivated by the following three questions:

1. Does MYB75 function in regulation of secondary cell wall formation and carbon redistribution in the Arabidopsis inflorescence stem?

MYB75 is a known regulator of the anthocyanin branch of the phenylpropanoid pathway in Arabidopsis, but how this regulation impact other aspects of carbon metabolism including formation of secondary cell wall is not known. Since the secondary cell wall represents one of the main carbon sinks in plants, and its formation requires coordination of metabolic fluxes through both polysaccharide and phenylpropanoid biosynthetic pathways, I hypothesized that transcription factors such as MYB75 could be involved in regulation and allocation of photosynthetic carbon among anthocyanin, lignin and other carbon metabolic pathways. To test this hypothesis, I examined secondary cell wall-enriched Arabidopsis stem tissue using loss-of-

function and gain-of function mutants to characterize the role of MYB75 in regulation of secondary cell wall formation.

2. Does MYB75 interact with other known or potential secondary cell wall transcription factors and if so, what is the role of such complexes in regulating secondary cell wall formation in the Arabidopsis inflorescence stem?

Protein-protein interactions are responsible for multi-protein complex formation among transcription factors, but how different protein complexes might regulate secondary cell wall formation in Arabidopsis stems is not apparent. My hypothesis is that MYB75 interacts with other secondary cell wall-regulating transcription factors and that the pattern of such protein interactions would reflect specific roles in regulating secondary cell wall biosynthesis in Arabidopsis inflorescence stems.

3. Do predicted protein complexes involved in regulating secondary cell wall formation in the Arabidopsis stem have conserved biological functions in seed coat formation?

Transcription factors execute their regulatory roles in ways that are modulated both spatially and temporally, but the role they play in different tissue types in the same organism is also conserved in many instances. My hypothesis is that transcription factor interaction(s) I identified and characterized in the Arabidopsis stem addressing Question 2 have conserved functional roles in other tissue types. As a system to test this hypothesis, I examined the development of the Arabidopsis seed coat, which involves deposition of thick secondary cell walls, to characterize the role of putative MYB75-associated protein complexes, and to identify novel interacting partners within this complex.

Chapter 2. MYB75 functions in regulation of secondary cell wall formation in the Arabidopsis inflorescence stem

2.1 SYNOPSIS

Deposition of lignified secondary cell walls in plants involves a major commitment of carbon skeletons in both the form of polysaccharides and phenylpropanoid constituents. This process is spatially and temporally regulated by transcription factors, including a number of MYB family transcription factors. MYB75, also called *PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT1*), is a known regulator of the anthocyanin branch of the phenylpropanoid pathway in Arabidopsis, but how this regulation impacts other aspects of carbon metabolism, especially secondary cell wall formation in Arabidopsis inflorescence stem is not known. This facet of MYB75 is being explored in this chapter.

2.2 INTRODUCTION

The allocation of carbon to different metabolic pathways in plants is a central feature of growth and development in plants (Bloom, et al., 1985; Smith and Stitt, 2007). However, the molecular underpinnings regulating the sensing and signaling system(s) that are anticipated to link carbon assimilation to particular metabolic pathways have yet to be identified. Plant secondary cell walls represent a major carbon sink in plants (Brown, et al., 2005; Pauly and Keegstra, 2008) and many proteins catalyzing deposition of secondary cell wall polysaccharides and lignin have been characterized (Zhong and Ye, 2007). For example, the synthesis of cellulose at the plasma membrane during both primary and secondary cell wall synthesis has been shown to be dependent on distinct CESA (Cellulose Synthase A) proteins. The deposition of cellulose in the secondary cell wall involves CESA4, 7 and 8 in Arabidopsis (Persson, et al., 2005; Brown, et al., 2009; Brown, et al., 2005; Somerville, et al., 2004), while primary cell wall cellulose formation is orchestrated by CESA3, 6 and 9 (Joshi and Mansfield, 2007). Additional polysaccharide components are synthesized by enzymes involved in hemicellulose production, primarily of the

glycosyltransferases (GT) families (Persson, et al., 2007; Brown, et al., 2009). Secondary cell wall lignin biosynthesis requires the activity of both core phenylpropanoid pathway enzymes such as L-phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H), as well as enzymes more directly engaged with lignin biosynthesis, including caffeoyl-CoA *O*-methyltransferase (CCoAOMT) (Do, et al., 2007), ferulate 5-hydroxylase (F5H)(Meyer, et al., 1998), cinnamoyl-CoA reductase 1, CCR1 (Mir Derikvand, et al., 2008) and cinnamyl alcohol dehydrogenase (CAD)(Sibout, et al., 2005). The regulation of the corresponding genes is in part facilitated by a number of transcription factors (TFs) which have been shown to regulate secondary cell wall formation in Arabidopsis and other plants, and include members of the NAC domain (Kubo, et al., 2005) and MYB family of transcription factors (Zhong and Ye, 2007; Zhong and Ye, 2009).

Lignin biosynthesis represents a major terminal product of phenylpropanoid metabolism, a multi-branched system of reactions that converts the carbon skeleton of L-phenylalanine into a wide variety of phenolic plant metabolites. Another major branch of phenylpropanoid metabolism generates flavonoids, a diverse group of phenolics that includes flavonols, isoflavonoids, leucoanthocyanidins (tannins) and anthocyanins. Various transcription factors, including a number of MYB family proteins, have been shown to directly or indirectly regulate the activity of genes encoding enzymes involved in specific branches of the phenylpropanoid pathway, (Zhong and Ye, 2007; Zhong and Ye, 2009; Davies and Schwinn, 2003; Dubos, et al., 2005; Gonzalez, et al., 2008), but less is known about the transcriptional regulation of carbon partitioning across different branches of phenylpropanoid metabolism by individual transcription factors.

MYB75 (At1g56650), also known as *PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT1*) was earlier identified as a positive regulator of anthocyanin biosynthesis in Arabidopsis, based on the strong accumulation of anthocyanins in activation-tagged seedlings over-expressing the *MYB75/PAP1* gene (Borevitz, et al., 2000; Pourtau, et al., 2006; Gonzalez, et al., 2008). In addition to this impact of PAP1 over-expression on anthocyanin production, the activity of *PAP1*/*MYB75* has been found to influence senescence (Bernhardt, et al., 2003), sucrose signaling (Teng, et al., 2005), and lignin deposition in Arabidopsis (Borevitz, et al., 2000). A loss-of-function *MYB75* allele (*myb75-1*) harbors mutations inside the DNA-binding domain of the encoded protein (Teng, et al., 2005) and *myb75-1* plants show only a very weak

anthocyanin accumulation response to elevated levels of sucrose. The expression of the MYB75 gene is also responsive to a number of abiotic factors, including nitrogen deficiency, phosphate starvation, high temperature and high light intensity (Rowan, et al., 2009; Lea, et al., 2007; Misson, et al., 2005; Tohge, et al., 2005b; Tohge, et al., 2005a; Morcuende, et al., 2007; Muller, et al., 2007; Vanderauwera, et al., 2005), and this spectrum of metabolic and environmental sensitivities suggests that, in addition to modulating anthocyanin biosynthesis, MYB75 might play a more general role in regulating cellular metabolism.

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

2.3 MATERIALS AND METHODS

2.3.1 Plant material

The Arabidopsis loss-of-function allele of MYB75 (*myb75-1*) (pst16228) is the result of a *Ds* insertion at the *MYB75* locus, in the Nossen ecotype background (Kuromori, et al., 2004) and was obtained from the RIKEN Bioresource Centre (http://rarge.gsc.riken.jp/dsmutant/index.pl. This allele has been genetically characterized earlier and demonstrated to possess a *Ds* insertion tightly linked to the MYB75 phenotype (Teng, et al., 2005). I further confirmed this insertion in the *MYB75* locus and identified plants homozygous for this insertion by PCR screening. For genotyping of the homozygous insertion lines, primer sequences used were: left primer (LP), 5′-

TGGTTTTGTAGGGCTAAACCG-3′ and right primer (RP), 5′-AAACACCGGATACATACCTTTTTC-3′. When the fragment was amplified using LP with ds5-3 (5′-TACCTCGGGTTCGAAATCGAT-3′) and RP with ds3-2a (5′-CCGGATCGTATCGGTTTTCG-3′), the wild-type produced a PCR product of approximately 900 bp (from LP to RP). Lines carrying the homozygous insertion produced an approximate 500 bp band (from RP to ds 3-2a), while heterozygous lines produced both bands. The PCR amplification program used for this analysis used a cycle of 94°C for 2 min; 30 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 3 min followed by 72°C for 10 min.

The gain-of-function mutant for *MYB75* (*MYB75(*o/x)) (Borevitz, et al., 2000) is an activation-tagged mutant *(pap-1D*) in the Columbia background, and was obtained from the Arabidopsis Biological Resource Center. Homozygous plants of each genotype were used for all experiments, along with appropriate wild type plants (*myb75-1* vs. Nossen and *pap1-D* vs. Columbia) as comparison controls.

Seeds were surface-sterilized using 20% commercial bleach, cold-treated at 4°C in the dark for 2 days and plated on ½ MS agar medium (2.16 g/l MS salts, 1% sucrose, 1% Bacto-agar pH 6.0 adjusted with 1 M KOH (Murashige and Skoog, 1962). Ten-day-old seedlings were grown in a moistened Sunshine Mix #1 (Sun Gro Horticulture Canada Ltd),with a 16/8 h (light/dark) photoperiod at approximately 120 µmol m^{-2} sec^{-1} and a temperature of 23°C, unless specified otherwise. Growth comparisons were performed on 6-week-old plants grown under the above described conditions. For TEM, microscopy and qRT-PCR experiments, inflorescence stems were harvested from eight-week-old plants and the lower half of the stem was used for analysis. For chemical analyses, whole stems were dried in a 50°C oven overnight and ground in a Wiley mill to pass a 40-mesh screen.

2.3.2 Analysis of anthocyanin content

Seedling anthocyanin content was determined using a procedure modified from that of Neff and Chory (1998). In short, at least two groups (50 seedlings each) of eight-day-old seedlings from each genotype were extracted overnight in 150 µl methanol which was acidified with 1% HCl. After the addition of 100 µl distilled water and 250 µl chloroform, anthocyanins were separated from chlorophylls by solvent partitioning. Total anthocyanin content in the aqueous phase was

determined spectroscopically by measuring the A_{530} and A_{657}. By subtracting the A_{657} from the A_{530}, the relative amount of anthocyanin per seedling was calculated [(A530 − A657) × 50 seedling^{-1}].

2.3.3 *GUS* reporter gene analyses

Transgenic Arabidopsis plants (T_2) generated earlier by Gonzalez et al. (2008) were employed in this study (gift from Dr. A.M. Lloyd (University of Texas at Austin, TX). They express a *MYB75pro::GUS* construct derived from the 2.2 kb genomic DNA region upstream of the MYB75 coding sequence in the Columbia ecotype, fused with the *beta*-glucuronidase coding sequence. Histochemical analysis of the GUS reporter gene expression was performed as described previously (Malamy and Benfey, 1997) using different plant organs, as well as transverse hand sections of inflorescence stems of 6-8-week-old T_2 *MYB75pro::GUS* transgenic plants.

2.3.4 Protoplast isolation, transfection and GUS activity assay

For protoplast isolation, leaves from 3–4 weeks old Columbia wild type plants were used and subsequent transfection and GUS activity assays were performed as described previously (Wang, et al., 2007). The plasmid DNAs for reporter and effector genes were isolated using Endofree Plasmid Maxi Kits (Qiagen, Mississauga, Ontario, Canada). Both effector plasmid (10 µg) and reporter plasmid (10 µg) were co-transfected and all assays were performed in triplicate.

2.3.5 Localization of MYB75-YFP

To examine the subcellular localization of MYB75–YFP in protoplasts, the Gateway recombination cassette (Invitrogen) from pEARLYGATE104 (Earley, et al., 2006) was used as a destination vector for generation of an N-terminal YFP fusion of MYB75. The resulting plasmid was transfected into freshly prepared Arabidopsis leaf mesophyll protoplasts, and incubated for 20–22 hours (Wang, et al., 2007). YFP fluorescence was visualized using a Leica DM-6000B fluorescence microscope and photographed with a Leica digital image system (Leica Microsystems).

2.3.6 qRT-PCR

For organ-specific expression analyses, total RNA was isolated from different organs of 6-week-old Arabidopsis (Nossen WT) plants (three biological replicates, each consisting of pooled tissues from 8-10 plants) and qPCR was performed as described below. Relative values are arbitrary units and were calculated as described previously (Gutierrez, et al., 2008). For the secondary cell wall-specific and lignin-specific gene expression study, total RNA for real-time PCR was isolated from the lower half of inflorescence stems (WT, *myb75-1* and *MYB75*(o/x)) with three biological replicates (each consisting of pooled stems from 8-10 plants) using the RNeasy Mini Protocol (Qiagen). To eliminate residual genomic DNA, the RNA was treated with RNAse-free DNAseI according to the manufacturer's instructions (Qiagen). The concentration of RNA was quantified using the absorbance at 260 nm and the quality was assessed using the A260/A280 ratio. Total RNA (2 µg) was reverse transcribed using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. cDNA was diluted (1:20) and 2 µl was used in each reaction in a 20 µl reaction volume. PCR amplification was performed with gene-specific primers for secondary cell wall or lignin-specific genes (Table S1), using *Actin8* as a normalization control (Table S1). *Actin8* was chosen as the reference gene for this study as its expression has been shown to be relatively invariant (An, et al., 1996). However, in my experiments, stable expression of *Actin8* was reconfirmed in inflorescence stem samples (data not shown). The cDNA was amplified using the PerfeCta™ qPCR FastMix (Quanta Biosciences) on the DNA Engine Opticon® 2 (Bio-Rad). Differences in gene expression, expressed as fold-change relative to control, were calculated using the [delta][delta]C_t = 2 $^{[delta]Ct,Actin - [delta]Ct,gene}$ method. Each measurement was carried out in triplicate, and the error bars represent standard error of the mean (SEM) of fold-changes for the three biological replicates.

2.3.7 Bright field and transmission electron microscopy

Tissue for light and transmission electron microscopy (TEM) was harvested from the inflorescence stem (5 cm from the base) of 8-10-week-old plants and fixed with glutaraldehyde. Fixed stems were vacuum infiltrated in 1% osmium tetroxide, 0.05M sodium cacodylate (pH 6.9) for 30 minutes, rinsed twice and then dehydrated through an aqueous alcohol series (30-100%; 15

minutes for each dilution). Dehydrated stems were soaked twice in anhydrous acetone before embedding in low viscosity Spurr's resin. Sections (0.5 μm) were cut using a Leica Ultracut T and Druuker diamond Histoknife, and stained with Toluidine Blue for bright field microscopy (using an Olympus AX70 microscope) or with uranyl acetate for TEM (viewed on a Hitachi H7600 PC-TEM).

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

2.3.8 Chemical analysis

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

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

2.3.9 Microarray analysis

Total RNA was extracted from Arabidopsis inflorescence stems (Nossen wild type and *myb75-1*) using a QIAGEN Plant Mini RNA extraction kit (Qiagen Inc.). The quantity and quality of total RNA were assessed on the Agilent 2100 Bioanalyzer (Agilent Technologies) using the Agilent RNA 6000 Nano kit and reagents. Samples of total RNA (10 μg) for six WT and six *myb75-1* biological replicates were reverse transcribed using a SuperScript II RT kit (Invitrogen) and the

appropriate 3DNA primers (cyanine5- or cyanine3-specific capture sequences) to achieve dye balance with two technical replicates for each of three biological replicate pairings. The 3DNA Array 350 kit (Genisphere) was used according to manufacturer specifications for cDNA hybridizations, and subsequent 3DNA (dendromer) fluorescent probe hybridizations onto custom-made full-genome (30K) Arabidopsis 70-mer oligo arrays (Ehlting, et al., 2005; Douglas and Ehlting, 2005) printed at the Prostate Centre Microarray Facility, Vancouver, Canada. Hybridizations were carried out using a Slidebooster SB401 (Advalytix) according to Array 350 specifications and the hybridized slides were scanned with a ScanArray Express (PerkinElmer). Scanned images were quantified using Imagene software (BioDiscovery), and the resulting data were analyzed in the R package using Bioconductor tools and custom scripts. For background correction, the mean of the dimmest five percent of spots in a particular sub-grid (grouping of 26 x 27 spots) was used as the background value for the spots in that sub-grid. Background-corrected spot intensities were then normalized on each array using the robust local-linear regression algorithm LOWESS (or LOESS) included in the R package, with a span of 0.7 (Yang, et al., 2002). The relative expression ratio for each gene represents the average of three biological replicates, where p-value significance estimates were computed using a two-tailed Students t-tests (alpha = 0.05) and adjusted for false discovery rate using a q-value correction based upon Storey (2002).

2.3.10 Primers

Table 2.1 Primers used in this study (Chapter 2).

Gene ID	Name	Primer sequence (5'-3')
At1g56650	MYB75	MYB75-L: TGGCACCAAGTTCCTGTAAG
		MYB75-R: AAGCCTATGAAGGCGAAGAA
At2g37040	PAL1	PAL1-L: AAGATTGGAGCTTTCGAGGA
		PAL1-R: TCTGTTCCAAGCTCTTCCCT
At2g30490	C4H	C4H-L: ACTGGCTTCAAGTCGGAGAT
		C4H-R: ACACGACGTTTCTCGTTCTG
At1g51680	4CL1	4CL1-L: TCAACCCGGTGAGATTTGTA
		4CL1-R: TCGTCATCGATCAATCCAAT
At5g48930	HCT	HCT-L: GCCTGCACCAAGTATGAAGA
		HCT-R: GACAGTGTTCCCATCCTCCT
At2g40890	C3H1	C3H1-L: GTTGGACTTGACCGGATCTT
		C3H1-R: ATTAGAGGCGTTGGAGGATG
At4g34050	CCOMT 1	CCOMT1-L: CTCAGGGAAGTGACAGCAAA
		CCOMT1-R: GTGGCGAGAAGAGAGTAGCC
At1g15950	CCR1	CCR1-L: GTGCAAAGCAGATCTTCAGG
		CCR1-R: GCCGCAGCATTAATTACAAA
At4g36220	F5H	F5H-L: CTTCAACGTAGCGGATTTCA
		F5H-R: AGATCATTACGGGCCTTCAC
At5g54160	COMT1	COMT1-L: TTCCATTGCTGCTCTTTGTC
		COMT1-R: CATGGTGATTGTGGAATGGT
At4g34230	CAD5	CAD5-L: TTGGCTGATTCGTTGGATTA
		CAD5-R: ATCACTTTCCTCCCAAGCAT
At4g32410	CesA1	CesA1-L: GGTATTTATTGCGGCAACCT
		CesA1-R: ATCCAACCAATCTCTTTGCC
At5g05170	CesA3	CesA3-L: ACAGCCAACACAGTGCTCTC
		CesA3-R: TGGTACCCATTTACGAGCAA
At2g21770	CesA6	CesA6-L: TGCCCTTGAGCACATAGAAG
		CesA6-R: GCACTCCACCATTTAGCAGA
At5g44030	CesA4	CesA4-L: GGATCAGCTCCGATCAATTT
		CesA4-R: ACCACAAAGGACAATGACGA
At5g17420	CesA7	CesA7-L: CAGGCGTACTCACAAATGCT
		CesA7-R: TGTCAATGCCATCAAACCTT
At4g18780	CesA8	CesA8-L: ACGGAGAGTTCTTTGTGGCT
		CesA8-R: GGTCTGTGTTGGAACAATGG
At5g54690	IRX8	IRX8-L: GTGGTCACAGGGAAAGGATT
		IRX8-R: AGCAAGAGAGGAGCAAGGAG
At2g37090	IRX9	IRX9-L: TTTGCGGGACTAAACAACAT
		IRX9-R: ATCGGAGGCTTTGTCTCTGT
At2g28110	FRA8	FRA8-L: GACTTGTTGAATCGGTGGCTC
		FRA8-R: GAAAGAGTTTGACCTTCTAAC
At5g60690	IFL1	IFL1-L: CCAAGCTGTGAATCTGTGGTC
		IFL1-R: CGATCTTTGAGGATCTCTGCA
At3g54890	LHCA1	At3g54890-L: GGTTTGACCCACTTGGACTT
		At3g54890-R: TGAGCCTTAACCCAGTTTCC
At1g61520	LHCA3	At1g61520-L: AGATGGCTCTGATGGGATTC
		At1g61520-L: GAGGGTTGAAGAAAGGTCCA
At5g54270	LHCB3	At5g54270-L: GGCTTCTTTGTTCAAGCCAT
		At5g54270-R: GGTGCAAACTTAGTTGCGAA
At1g45130	BGAL5	At1g45130-L: CTAAAGACGGTGGCTTGGAT
		At1g45130-R: AAGACCCACTTCCTGAATCG
At5g10430	AGP4	At5g10430-L: GCTCCTTCTCCTGCTGATGT
		At5g10430-R: AAAGCCTTGTTGGAGAATGC
At5g26000	BGLU38	At5g26000-L: AAGGCTTCATCTTCGGTGTT
		At5g26000-L: ATTTCCCAAATCAGCTCCAC
At1G49240	ACT8	Act8-L: TCTAAGGAGGAGCAGGTTTGA
		Act8-R: TTATCCGAGTTTGAAGAGGCTAC

2.3.11 Accession numbers

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

2.4 RESULTS

2.4.1 MYB75 expression in wild-type and in gain and loss –of- function mutants

To study the role of MYB75 in regulating cell wall biosynthesis and phenylpropanoid metabolism, an activation-tagged *MYB75* gain-of-function mutant (Figure 2.1A) (*pap1-D),* referred to here as *MYB75(o/x)*, was compared with a loss-of-function transposon-tagged *Ds* insertion mutant (*myb75-1*) (Figure 2.1A). Plants homozygous for both *MYB75 (o/x)* and *myb75-1* alleles were identified by PCR-aided genotyping and the abundance of *MYB75* transcripts was assessed using qRT-PCR. As expected, *MYB75* transcript abundance was higher in the over-expression line than in wild-type plants, while negligible *MYB75* expression was detected in the loss-of-function mutant (Figure 2.1B).

MYB75(o/x) seedlings showed elevated levels of anthocyanin accumulation under normal growth conditions, as reported earlier (Borevitz, et al., 2000), while the anthocyanin content was slightly reduced from wild type levels in *myb75-1* seedlings (Figure 2.1C), a pattern that is consistent with MYB75 acting as a positive regulator of this branch of the phenylpropanoid pathway. No visible difference in growth or inflorescence stem morphology was observed when the mutants were compared with their corresponding WT controls (Figure 2.1D) and grown under normal growth conditions.

When *MYB75* expression was assessed in various tissues of six-week-old plants by qRT-PCR, transcript levels were found to be highest in the lower part of inflorescence stem (Figure

2.2A). Lower levels of *MYB75* transcripts could be detected in flowers, leaves and siliques, but none could be detected in roots (Figure 2.2A).

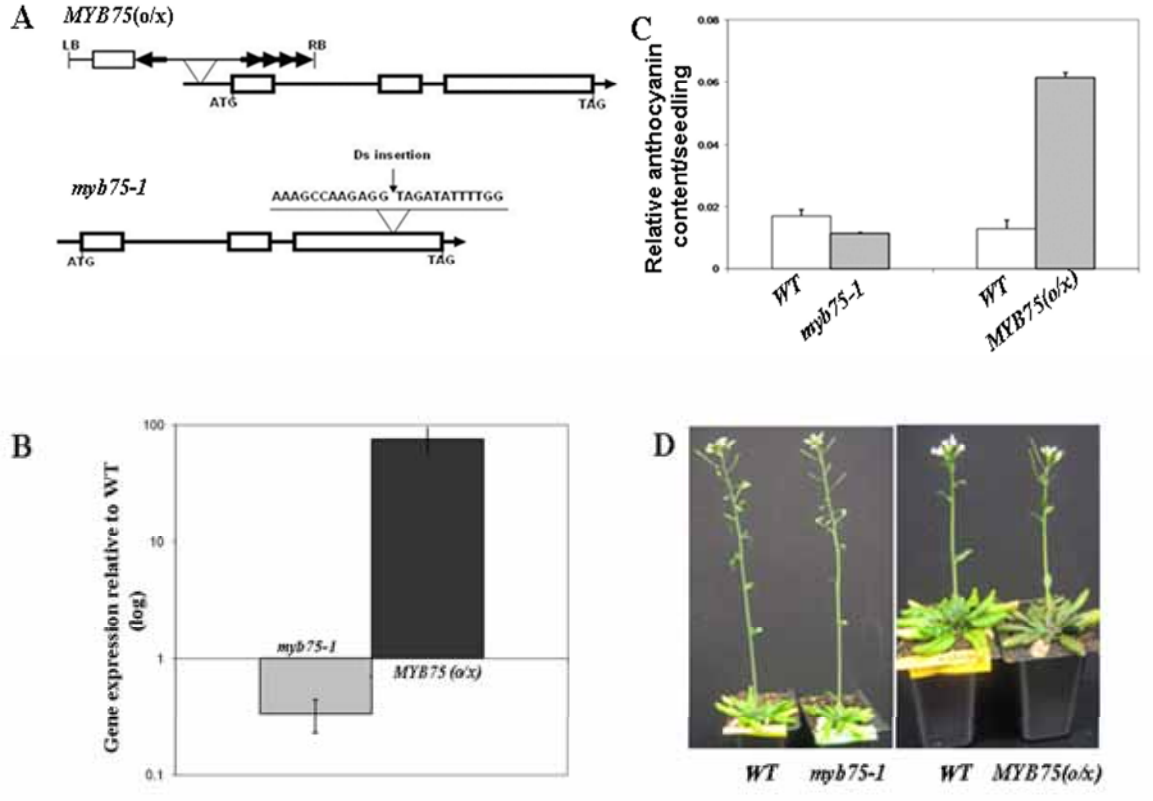


Figure 2.1 Activation-tagged and Ds insertion mutants of MYB75, anthocyanin content and growth and inflorescence stem phenotype. (A) Modified and adapted schematic diagram of *MYB75* gene and position of activation tag (MYB75 (o/x) (Borevitz et. al (2000)) or Ds insertion (*myb75*) (Kuromori et. al. (2004)). (B) qRT-PCR analysis of *MYB75* expression in *myb75* and *MYB75(o/x)*. Reduction in transgenic level is seen in *myb75-1* and up-regulation in *MYB75(o/x)*. Error bars represent SE of three biological replicates. (C) Comparison of anthocyanin accumulation in wild-type and the mutant seedlings (*myb75-1* and *MYB75(o/x)*. The anthocyanin is expressed as relative amount of anthocyanin per seedling. Bar represents SE of three independent replicates. (D) Comparison of growth and inflorescence stem phenotype in Nossen-WT vs. *myb75-1* and Columbia-WT vs. *MYB75(o/x)* in 6-weeks-old Arabidopsis plants.

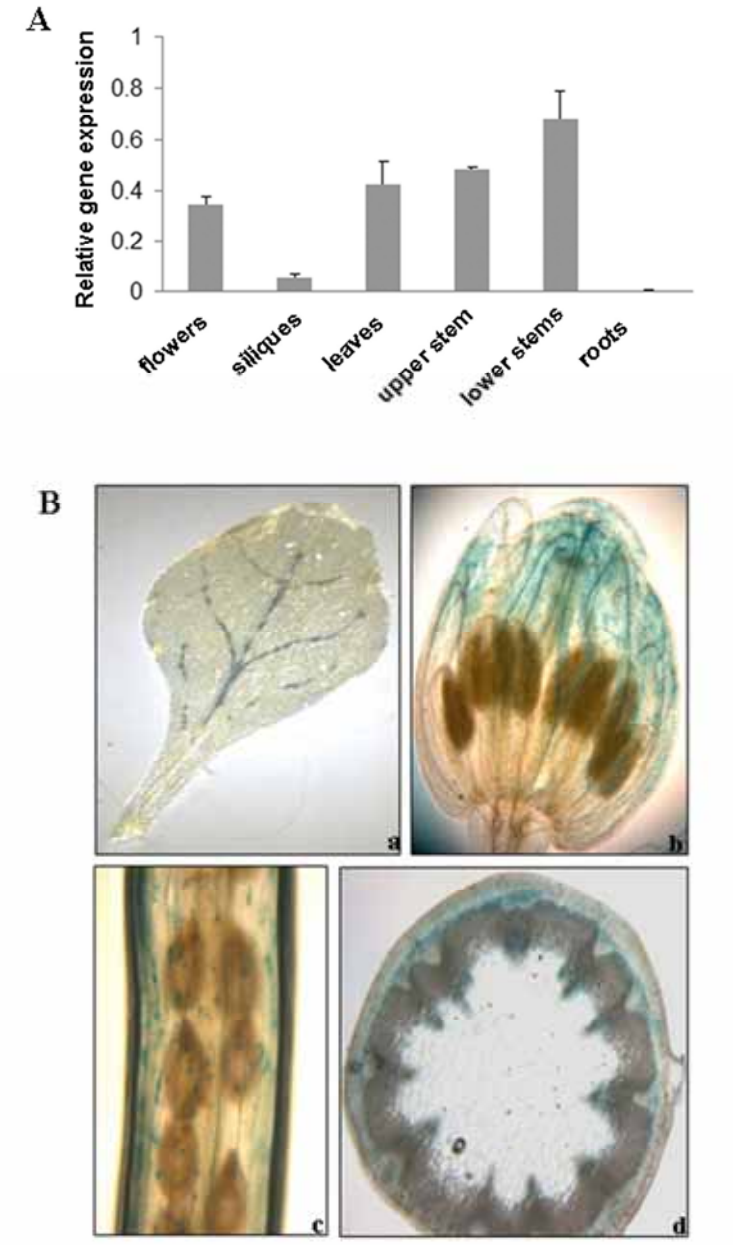


Figure 2.2 Expression pattern of the *MYB75* in mature Arabidopsis plant and stems.
(A) Quantitative PCR analysis showing the expression of *MYB75* in stems. The expression level of *MYB75* is relative to actin and is expressed in arbitrary units. Error bars represent SE of three biological replicates.(B) GUS expression pattern in different organs of mature *MYB75::GUS* plants (a) in leaf midrib (b) in the epidermal cells of the flower (c) in the epidermis of the silique (d) and in cortex and in vascular bundles in the cross-section of lower inflorescence stem.

In order to obtain a more refined spatially and developmentally resolved picture of the expression of *MYB75*, transgenic Arabidopsis plants containing a *MYB75pro::GUS* transgene were examined using histochemical staining for GUS activity (Figure 2.2B). It had been

previously reported that *GUS* expression is seen in most parts of *MYB75pro::GUS* seedlings (Gonzalez, et al., 2008), but in six-week-old plants we found that GUS activity was primarily localized in the vasculature of leaves and flowers (Figure 2.2B (a) and (b)), and in the epidermis of siliques (Figure 2.2B (c)), but not in roots (data not shown), a pattern consistent with the qRT-PCR data. Within the lower portion of the inflorescence stem, where the highest levels of *MYB75* transcript had been detected, GUS activity was observed specifically in the cortex, vascular bundles and fibers (Figure 2.2B (d)).

2.4.2 MYB75 is nuclear localized and acts as a transcriptional activator

An Arabidopsis protoplast transient expression system was used to assay the sub-cellular localization of a MYB75–YFP fusion protein, which was found to accumulate in the nucleus (Figure 2.3A). A protoplast transfection system (Wang, et al., 2007) was also employed to assess the transcriptional repression or activation activity of MYB75. Co-transfection of a *GAL4:GUS* reporter construct with an effector construct containing the MYB75 open reading frame fused to the GAL4 DNA-binding domain (GD) (Figure 2.3B) revealed that MYB75 could weakly activate expression of the GUS reporter gene being recruited to the promoter region of the reporter gene by GD (Figure 2.3C (a)).

To test the possibility that MYB75 might act as a transcriptional repressor, I co-expressed a construct containing the GUS gene driven by the CaMV35S promoter supplemented with both LexA and Gal4 DNA binding sites. When co-transfected with the MYB75-GD effector construct and the transactivator LD-VP16 (VP16 fused to LexA DNA-binding domain (LD)), the MYB75-GD gene product failed to suppress activation of the GUS reporter by LD-VP16. As a positive control, I also co-expressed the target reporter with a KNAT7-GD construct that had been previously shown function as a repressor (E. Li, S. Wang, J-G Chen, and C. J. Douglas, in preparation). As expected, KNAT7 expression strongly reduced GUS expression from the 35S/LexA/Gal4 *promoter::GUS* reporter (Figure 2.3C (b)). Taken together, these data suggest that, when acting alone, MYB75 may act as a weak transcriptional activator, but not as a repressor.

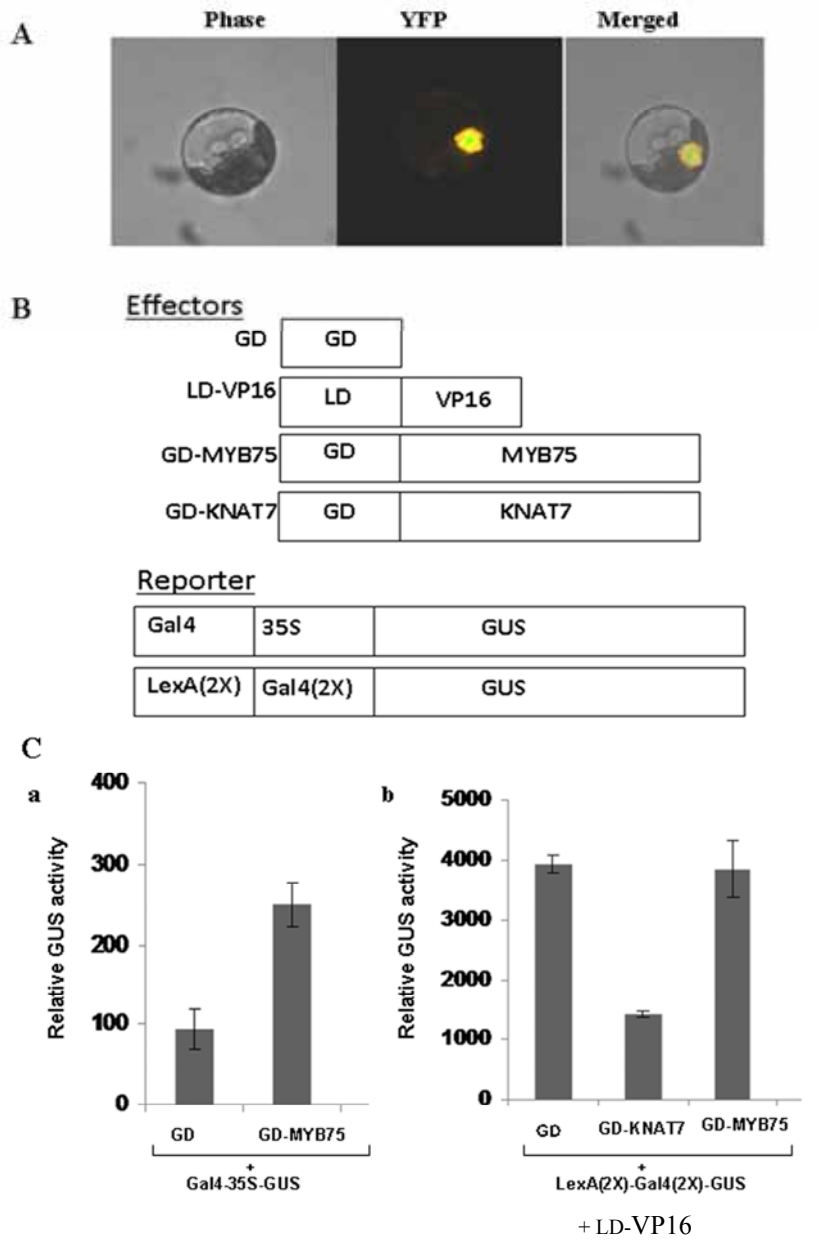


Figure 2.3 Nuclear localization and transcriptional activity of MYB75. (A) Protoplast transfected with 35S:MYB75-YFP. Left: DIC image; middle, YFP channel; right, merged images. (B) Effectors and reporter constructs used in the transfection assays. (C) Transcriptional activity of MYB75. Effector genes are the fusion of the *Ga14* DNA-binding domain (GD) in-frame with MYB75 or KNAT7. Effector gene plasmid DNA and reporter gene *Gal4: GUS* plasmid DNA were co-transfected into Arabidopsis leaf mesophyll protoplasts. GUS activity was assayed after the incubation of transfected protoplasts in darkness for 20–22 h. Shown are means ± SE of three replicates. (a) MYB75 is a weak transcriptional activator. (b) MYB75 does not repress the expression of the reporter activated by a transactivator.

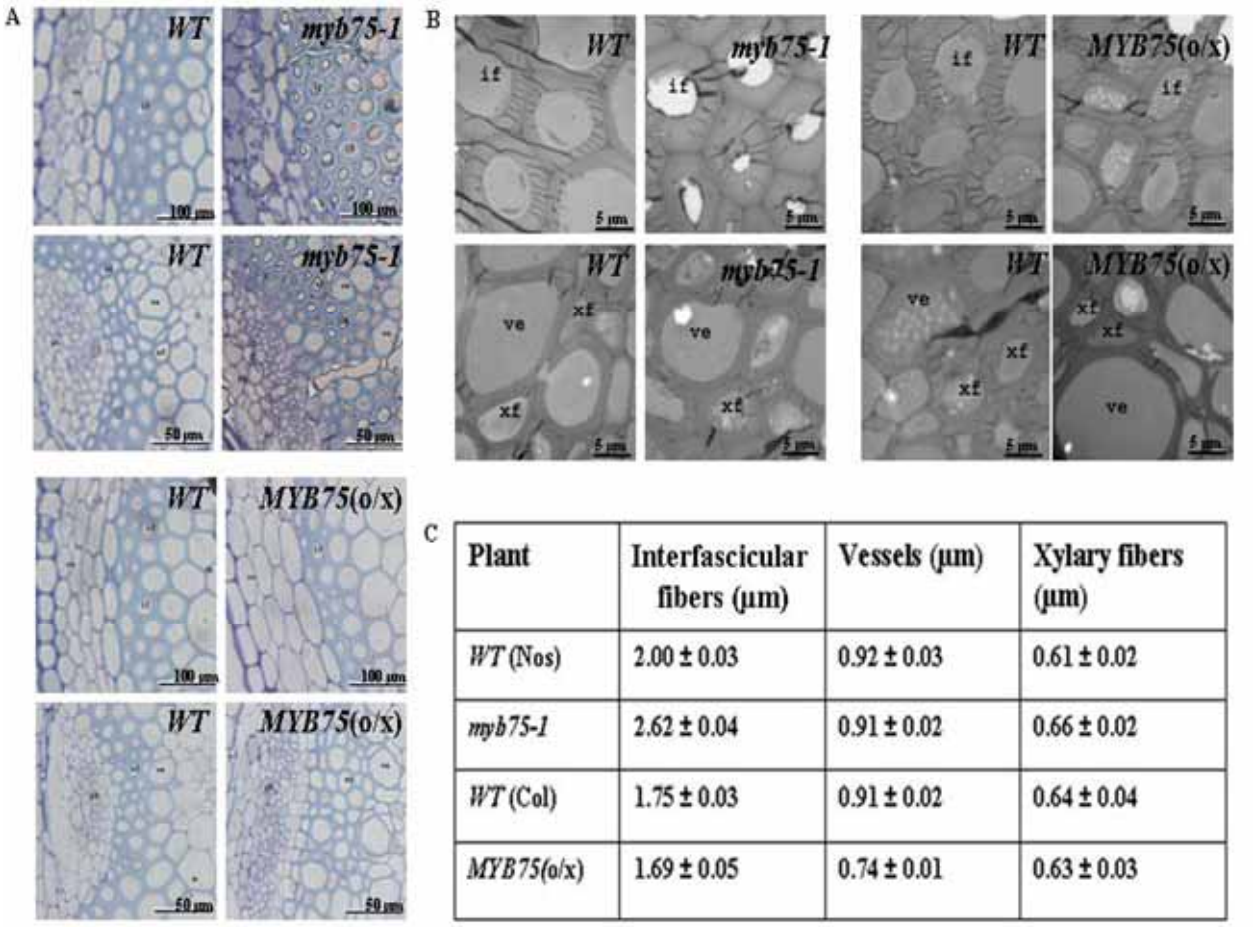
2.4.3 Loss of *MYB75* function affects secondary cell wall structure and composition

The RIKEN line pst16228 (*myb75-1* loss-of-function) has a transposon element insertion in the third exon of *MYB75* (Figure 2.1), and this Ds insertion event is linked to the phenotype of *myb75-1* as evident in the previous work (Teng, et al., 2005). No other loss-of-function alleles appear to be available, but a MYB75/PAP1-RNAi line has been reported to display an anthocyanin-deficient phenotype similar to that of pst16228 seedlings (Gonzalez, et al., 2008), confirming that this pigment phenotype is due to loss of *MYB75/PAP1* function.

When the basal portion of the inflorescence stem in *myb75-1* plants was examined in Toluidine Blue-stained cross sections (Figure 2.4A), and by transmission electron microscopy (Figure 2.4B), the secondary cell walls of the interfascicular fibers appeared to be thicker, compared with wild-type plants, while no change in vessel wall thickness or cell morphology was apparent (Figures 2.4A and 2.4B). Measurements taken from TEM micrographs confirmed that the interfascicular fiber wall thickness was increased in *myb75-1* plants, while little or no change was observed in vessel or xylary fiber wall thickness (Figure 2.4C). In the *MYB75(o/x)* plants, vessel thickness was significantly decreased adding to the possibility that MYB75 is a negative regulator of secondary cell wall formation in Arabidopsis stems. No obvious differences were observed in the primary cell walls.

To determine if these changes in interfascicular fiber wall thickness might be associated with changes in cell wall chemistry, the Klason lignin content in mature inflorescence stems of both loss-of-function and gain-of-function mutant plants was assayed. Klason lignin content was significantly greater in stems of *myb75-1* plants, but remained unaffected in the *MYB75(o/x)* genotype (Table 2.2). Thioacidolysis was used to estimate the relative amounts of syringyl (S) and guaiacyl (G) monomers in the inflorescence stem lignin. This analysis revealed that the S/G monomer ratio was lower in *myb75-1* plants as compared to the wild type, due primarily to lower levels of S subunits released by thioacidolysis (Table 2.3).

In the *MYB75(o/x)* genotype, the S/G ratio was higher than in wild type, due both to an increase in released S units, and a decrease in released G units (Table 2.2). When changes in cell wall carbohydrate content in both *myb75-1* and *MYB75(o/x)* lines were assayed, no significant changes in glucose content were observed, nor were significant differences detected in galactose, rhamnose, mannose or arabinose content in either mutant (Table 2.4).



C

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

Figure 2.4 Secondary wall thickening in interfascicular fibers and vessels in *myb75-1* and MYB75 (o/x) plants.The bottom inflorescence stem of Arabidopsis plants were used for assessment of secondary walls in fibers and vessels. (A) Toluidine Blue staining of the cross-sections in mutants and *WT* (Top; as indicated). Higher magnification of the Toluidine Blue stained sections (Lower panel; as indicated). *myb75-1* show thickened cell wall as compared to *WT*. (B) Transmission electron microscopy of wild type, *myb75-1* and *MYB75(o/x)*. *myb75-1* show thickened interfascicular fibers. (C) Measurements of secondary cell wall thickness in *WT* and mutants (as indicated) (µm)). The wall thickness was measured from transmission electron micrographs of fibers and vessels. Data are means (µm) ± SE from 75 cells. **if**, interfascicular fiber; **ve**, vessel; **xf**, xylary fiber. Bars = 50 µm and 100 µm (as indicated) in (A), and 5 µm in (B).

Table 2.2 Lignin content in the lower stems of the wild type and mutants	
Plant	**Lignin (mg/100mg)**
WT (Nos)	18.61 ± 1.01
myb75-1	23.24 ± 3.07
WT (Col)	21.09 ± 2.81
MYB75(o/x)	20.17 ± 2.89

Lignin content in the lower stems of the wild type and mutants as determined by Klason analysis. The analysis reveals a higher lignin content in *myb75-1* as compared to WT. Each data point is the mean (mg/100 mg dry cell walls) ± SE of two separate assays.

Table 2.3 Lignin composition in the lower stems of the wild type and mutants.			
	Monomer composition %		
Plant	**G lignin**	**S lignin**	**S/G**
WT (Nos)	55.61 ± 0.38	44.39 ± 0.40	0.78
myb75-1	58.94 ± 0.16	41.06 ± 0.18	0.67
WT (Col)	63.75 ± 0.18	36.25 ± 0.20	0.57
MYB75(o/x)	61.15 ± 0.56	38.85 ± 0.48	0.64

Lignin composition in the lower stems of the wild type and mutants as determined by thioacidolysis. Analysis show changes in S/G ratio in *myb75-1* as compared to WT. Each data point is the mean ± SE of four replicates.

Table 2.4 Cell wall composition in the lower stems of the wild type, and mutants determined by Klason analysis				
	***WT* (Nos)**	***myb75-1***	***WT* (Col)**	***MYB75*(o/x)**
Glucose	362.40 ± 1.77	371.80 ± 8.98	321.00 ± 14.84	291.70 ± 20.24
Arabinose	11.40 ± 3.56	12.20 ± 2.47	12.60 ± 1.65	10.80 ± 4.65
Xylose	104.00 ± 6.77	107.70 ± 2.29	97.10 ± 3.84	85.40 ± 8.67
Mannose	21.80 ± 5.50	20.20 ± 0.25	23.20 ± 5.63	21.70 ± 4.22
Rhamnose	9.40 ± 1.03	10.90 ± 3.53	13.4 ± 1.62	9.70 ± 1.50
Galactose	18.20 ± 1.10	18.40 ± 1.53	18.30 ± 1.97	18.50 ± 2.91

Cell wall composition in the lower stems of the wild type and mutants determined by Klason analysis. Each data point is the mean (mg/100mg dry cell walls) ± SE of two separate assays.

2.4.4 Loss of *MYB75* function affects expression of genes associated with secondary cell wall formation

Since manipulation of *MYB75* function led to changes in lignin content, I used qRT-PCR to examine the expression of genes encoding enzymes associated with phenylpropanoid metabolism and lignin monomer biosynthesis. This gene expression analysis focused on the lower inflorescence stems of the *myb75-1* and *MYB75(o/x)* lines, and compared these with the respective wild-type backgrounds. Many of the genes examined were found to be up-regulated in the *myb75-1* stems (Figure 2.5A), while their expression was generally unaffected in the *MYB75* over-expression background. I also assayed the expression of genes associated with cellulose and hemicellulose metabolism in secondary cell wall deposition or remodeling (Figure 2.5B). While expression of primary cell wall-associated cellulose synthase genes (*CesA3, CesA5* and *CesA6*) did not show any change in expression in either the loss-of function or gain-of-function mutant, genes encoding the cellulose synthase isoforms (CesA4, 7 and 8) believed to be specifically responsible for biosynthesis of secondary cell wall cellulose microfibrils (Taylor, et al., 2004) were strongly up-regulated in the *myb75-1* plant stems (Figure 2.5B).

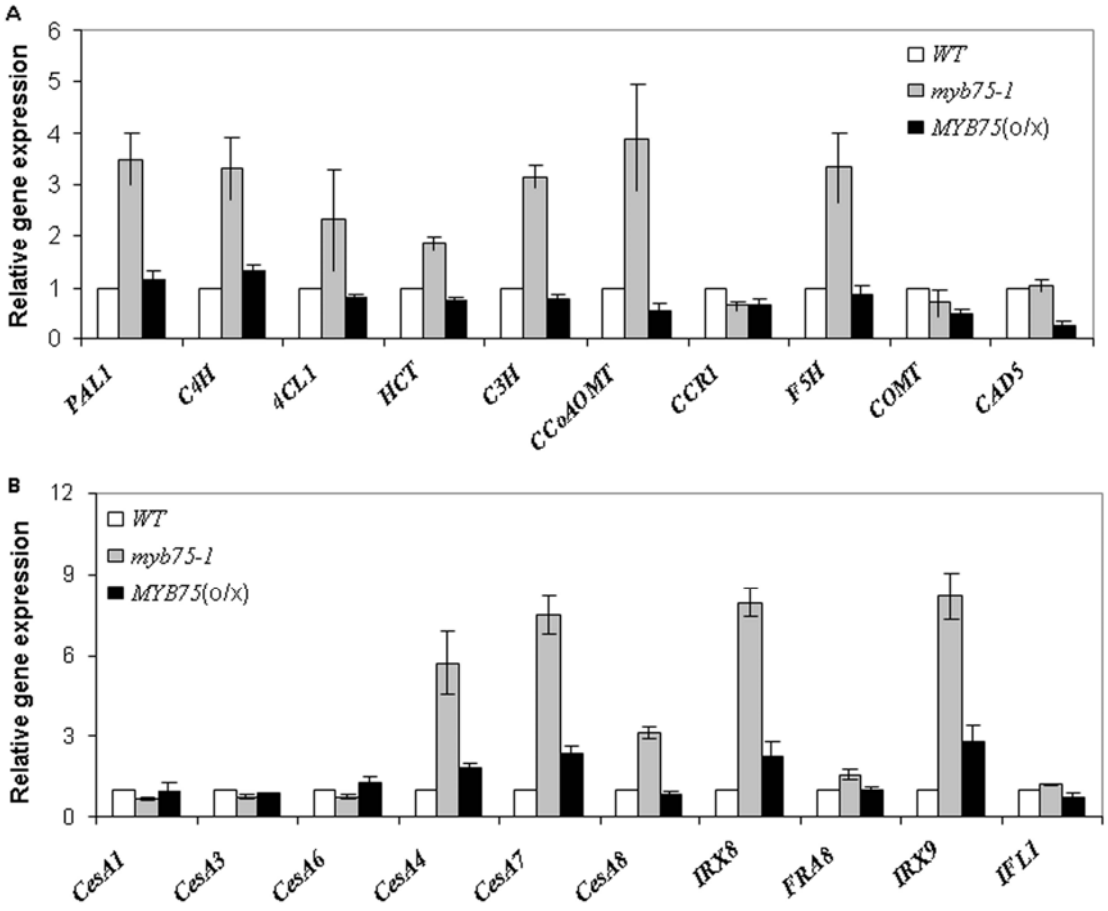


Figure 2.5 Secondary cell wall-associated gene expression in lower stems of myb75-1. (A) Real-time quantitative PCR analysis of the expression of lignin biosynthetic genes in lower inflorescence stems of *MYB75* over-expresser *(MYB75(o/x)*) and loss-of-function mutants (*myb75-1*) compared with the wild type. The expression levels of most of the genes in the lignin pathway were examined. PAL1, phenylalanine ammonia lyase 1; C4H, cinnamate 4-hydroxylase; 4CL1, 4-coumarate CoA ligase 1; HCT, hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyltransferase; C3H1, coumarate 3-hydroxylase 1; CCoAOMT1, caffeoyl CoA 3-*O*-methyltransferase 1; CCR1, cinnamoyl CoA reductase 1; F5H1, ferulate 5-hydroxylase 1; COMT1, caffeic acid *O*-methyltransferase 1; CAD5, cinnamyl alcohol dehydrogenase 5. (B) Expression of secondary wall biosynthetic genes in lower inflorescence stems of *MYB75* over-expresser (*MYB75(o/x)*) and loss-of-function mutants *(myb75-1*) compared with the wild type and real-time quantitative PCR analysis. The expression levels of genes involved in the biosynthesis of cellulose in primary cell wall (*CesA1, CesA3, CesA6*), cellulose in secondary cell wall (*CesA4*, *CesA7* and *CesA8*) and xylan (*FRA8, IRX8 and IRX9*), were examined. Error bars represent SE of three replicates.

In contrast to this impact on wall synthesizing systems, the expression of *IFL1*, a gene regulating interfascicular fiber differentiation in Arabidopsis (*IFL1)*, or of putative

glucuronyltransferase, *FRA8,* did not show any changes relative to wild type stems. Interestingly, however, expression of two xylan biosynthetic genes, *IRX8* and *IRX9* (Persson, et al., 2007; Pena, et al., 2007) was increased in *myb75-1* (Figure 4B). No reciprocal pattern of reduced gene expression of either phenylpropanoid or *CesA* genes was observed in the *MYB75* over-expression plants, relative to wild type plants (Figure 2.5).

To verify the observations reported earlier in literature for *MYB75(o/x)* (Borevitz, et al., 2000; Tohge, et al., 2005b), qRT-PCR was performed to examine the expression of genes encoding enzymes associated with phenylpropanoid metabolism and lignin monomer biosynthesis in eight-day-old seedlings of this line (Figure 2.6). This gene expression profile proved to be different from that which I had seen in the lower inflorescence stems of the *MYB75(o/x)* line, and was similar to the pattern that had been observed in previous studies of whole plants (Borevitz, et al., 2000; Tohge, et al., 2005b).

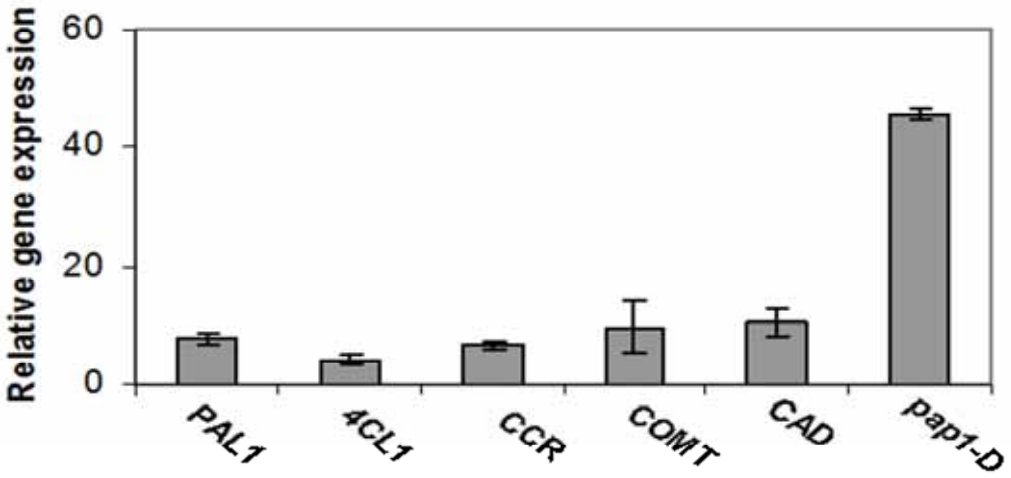


Figure 2.6 Real-time quantitative PCR analysis of the expression of lignin biosynthetic genes in seedlings of MYB75 over-expresser (*MYB75(o/x))* compared with the wild type. The expression levels of most of the genes in the lignin pathway were examined. *PAL1*, phenylalanine ammonia lyase 1; *4CL1*, 4-coumarate CoA ligase 1; *CCR1*, cinnamoyl CoA reductase 1; *COMT*, caffeic acid *O*-methyltransferase; *CAD*, cinnamyl alcohol dehydrogenase. Error bars represent SE of three replicates.

2.4.5 Photosynthetic machinery and cell wall modification genes are up-regulated in the *myb75-1* loss-of-function mutant

To obtain a broader perspective on the possible role of MYB75 in carbon redistribution during inflorescence stem development in Arabidopsis, I used microarray profiling to compare the global gene expression profiles of WT and *myb75-1* plants, with a specific focus on transcriptional activity in the maturing inflorescence stem. This analysis revealed that loss of MYB75 function resulted in both up-regulation and down-regulation of different gene sets (Table 2.6). Application of stringent cut-off values in the microarray analysis ($p<0.05$ and fold-change >2) excluded some secondary cell wall genes that had been earlier shown by qRT-PCR to be up-regulated in the *myb75-1* genotype (Figure 2.6), but the filtered microarray gene lists still included a number of up-regulated genes whose products are either predicted to be involved in cell-wall modification, or are important in other aspects of carbon metabolism, such as photosynthesis (Table 2.5).

Table 2.5: Expression changes of genes related to the photosynthetic machinery and cell wall modification in inflorescence stem of *myb75-1*

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

Table 2.6 Genes up-regulated or down-regulated in WT vs. *myb75* microarray study. P<0.05.

Down-regulated genes

GeneID	Annotation	Fold change	*p*-values
At1g76960	Expressed protein	-3.65	0.017
At5g52100	Dihydrodipicolinate reductase-related protein	-2.60	0.046
At1g46840	F-box protein family	-2.23	0.019

Up-regulated genes

GeneID	Annotation	Fold change	*p*-values
At3g48560	Acetolactate synthase	7.70	0.012
At3g54890	Light-harvesting chlorophyll a/b binding protein	5.31	0.016
At1g29910	Photosystem II type I chlorophyll a /b binding protein	4.05	0.016
At1g55330	Arabinogalactan-protein (AGP21)	3.89	0.049
At1g61520	Light-harvesting chlorophyll a/b binding protein	3.38	0.006
At5g54270	Light-harvesting chlorophyll a/b binding protein	3.07	0.036
At5g38420	RuBisCO small subunit 2b	2.89	0.003
At3g54890	Light-harvesting chlorophyll a/b binding protein	2.81	0.010
At3g53420	Plasma membrane intrinsic protein 2A	2.72	0.029
At1g77480	Nucellin protein, putative	2.69	0.018
At3g26520	Tonoplast intrinsic protein, putative	2.63	0.045
At3g51600	Nonspecific lipid transfer protein 5 (LTP 5)	2.61	0.038
At1g45130	Glycosyl hydrolase family 35 (beta-galactosidase)	2.52	0.048
At1g67090	Ribulose-bisphosphate carboxylase small unit -related	2.51	0.027
At4g23400	Major intrinsic protein (MIP) family	2.38	0.029
At3g50000	Casein kinase II alpha chain 2	2.36	0.037
At4g01150	Expressed protein	2.28	0.040
At5g10430	Arabinogalactan-protein (AGP4)	2.27	0.028
At2g35890	Calcium-dependent protein kinase(CDPK)	2.25	0.036
At5g26000	Glycosyl hydrolase family 1, myrosinase precursor	2.21	0.046
At1g05135	Expressed protein	2.17	0.009
At1g35850	Pumilio-family R-binding protein, putative	2.17	0.028
At5g59740	Protein serine /threonine kinase - like protein	2.16	0.029
At1g22275	Hypothetical protein	2.15	0.006
At3g60740	Tubulin folding cofactor D	2.14	0.037
At3g05830	Expressed protein	2.13	0.009
At1g61520	Light-harvesting chlorophyll a/b binding protein	2.12	0.017
At1g62840	Hypothetical protein	2.10	0.035
At1g07280	Hypothetical protein	2.03	0.045
At1g65930	Isocitrate dehydrogenase	2.03	0.009
At4g34620	Ribosomal protein S16p family	2.01	0.037

contd……...

contd……

Genes associated with secondary cell wall formation

GeneID	Annotation	Fold change	p-values
At2g37040	phenylalanine ammonia lyase (PAL1)	1.23	0.016
At3g10340	phenylalanine ammonia-lyase (PAL4),	1.72	0.049
At2g30490	cinnamate-4-hydroxylase (C4H)	2.01	0.018
At1g51680	4-coumarate:CoA ligase 1 (4CL1)	1.01	0.028
At3g21240	4-coumarate:CoA ligase 2 (4CL2)	1.14	0.036
At5g48930	hydroxycinnamoyltransferase (HCT)	1.11	0.046
At2g40890	cytochrome P450 98A3 (C3H)	1.28	0.009
At4g34050	caffeoyl-CoA 3-O-methyltransferase	1.57	0.028
At4g36220	ferulate-5-hydroxylase (F5H) (FAH1) (CYP84A1)	1.17	0.029
At5g54160	O-methyltransferase 1 (COMT)	1.41	0.006
At4g34230	cinnamyl-alcohol dehydrogenase (CAD1)	1.40	0.009
At4g32410	cellulose synthase, catalytic subunit,(CesA1)	1.02	0.028
At5g05170	cellulose synthase, catalytic subunit (CesA3)	1.07	0.029
At5g64740	cellulose synthase, catalytic subunit (CesA6)	1.04	0.046
At5g44030	cellulose synthase (CesA4)	2.71	0.009
At5g17420	cellulose synthaset(IRX3)(CesA7)	1.35	0.028
At4g18780	cellulose synthase (IRX1) (CesA8)	1.83	0.029
At5g54690	glycosyltransferase family 8 (IRX8)	1.33	0.006
At2g37090	glycosyltransferase family 43 (IRX9)	1.76	0.037
At4g36890	glycosyltransferase family 43(IRX14)	1.16	0.009
At4g30270	xyloglucan endotransglycosylase (meri5B)	1.81	0.017
At2g06850	xyloglucan endotransglycosylase (ext/EXGT-A1)	1.52	0.035

These include glycosyl hydrolases, and a putative arabinogalactan protein, several members of the light harvesting protein complexes, LHCA1 (At3g54890), LHCB1 (At1g29910), LHCA3 (At1g61520), LHCB3 (At5g54270) and also genes encoding ribulose-biphosphate carboxylase small subunit (At5g38420 and At1g67090). Differential expression of these genes was subsequently validated by qRT-PCR analysis (Figure 2.7).

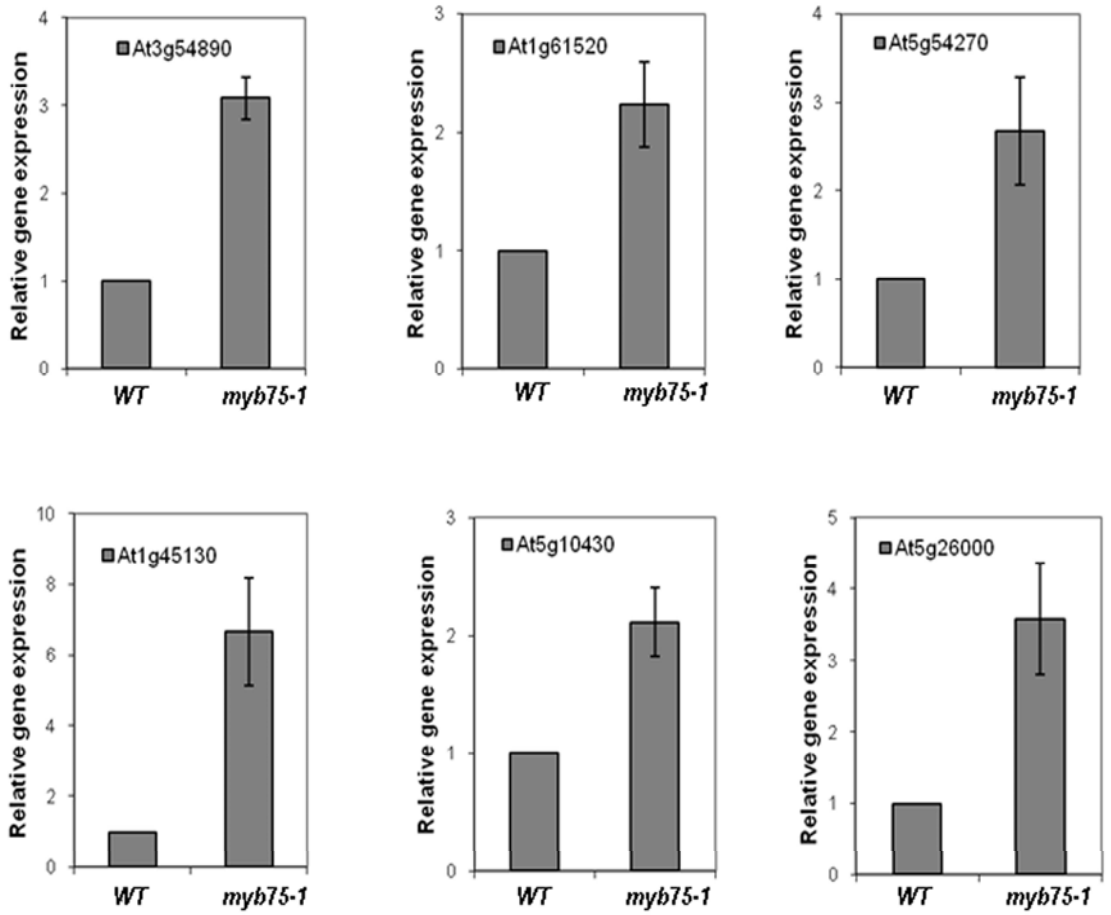


Figure 2.7 qRT-PCR validation of expression of candidate genes identified by microarray analysis to be up-regulated (> 2 folds; p, 0.05, Table 1) in the lower inflorescence stem of myb75, relative to Nossen-WT. Error bars represent ± SD of two biological replicates.

2.5 DISCUSSION

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

it impacts the lignin branch, in particular. The regulation of cell wall deposition is especially relevant for the inflorescence stems in Arabidopsis because of the prominence in the mature stem of interfascicular fibers and xylem vessels that possess lignified secondary cells walls.

MYB75 was originally characterized as a transcriptional regulator promoting anthocyanin biosynthesis (Gonzalez, et al., 2008) and much of that earlier work focused on phenotypes in seedlings and other juvenile tissues that display only limited commitment to secondary wall formation. It appears from my *MYB75* expression data that, as the plant matures, the ubiquitous *MYB75* expression pattern reported previously in juvenile vegetative tissues becomes restricted to specific tissues as development proceeds. This observation is also consistent with the *MYB75* expression data from AtGenExpress (Schmid, et al., 2005).

Cellulose synthesis in primary and secondary cell walls in Arabidopsis is believed to rely upon distinct members of the *CesA* gene family (Brown, et al., 2005). A *MYB75* loss-of-function mutant displayed no obvious defects in primary cell wall formation, but instead showed changes in the thickness of interfascicular fiber secondary cell walls and in cell wall chemistry of inflorescence stems in which secondary walls predominate. Consistent with a specific role for *MYB75* in the regulation of secondary cell wall biosynthesis, I observed *MYB75*-dependent regulation of a set of lignin biosynthetic genes (Figure 2.5A), as well as those *CesA* genes thought to be dedicated to secondary cell wall synthesis (Figure 2.5B).

Some secondary cell wall–associated MYB transcription factors such as Arabidopsis MYB4 and MYB32 have been previously shown to act as negative transcriptional regulators of at least some steps in the phenylpropanoid pathway (Preston, et al., 2004). MYB4 is closely related to MYB32 and in an MYB32 loss-of-function mutant, the transcript levels of the *Dihydroflavonol-4-reductase (DFR)* and *Anthocyanidin synthase (ANS)* genes decreased while the level of the COMT transcript increased. This indicates that MYB32 and MYB4 may influence the flux along the phenylpropanoid pathways, affecting the composition of the pollen wall.

Although loss of MYB75 function in the *myb75-1* mutant resulted in activation of genes encoding enzymes involved in lignin and cellulose biosynthesis, accompanied by an increase in interfascicular fiber cell wall thickness, our test of MYB75 *trans*-activation activity in a protoplast reporter system demonstrated that MYB75 displays only weak transcriptional activator activity and does not have repressor activity by itself within this assay system. The weak

transcriptional activation activity, or lack of transcriptional repression activity, of MYB75 in the *trans*-action reporter assays employed in this study could be due to the absence from mesophyll protoplasts of appropriate interaction partners that affect MYB75 function. In addition, KNAT7 repression observed in this system could, in principle, be due to steric hindrance, rather than representing genetic regulation. Alternatively, MYB75 could be required for the activation of unknown downstream regulators that themselves function to repress expression of genes encoding secondary cell wall biosynthetic genes. The effect of altered *MYB75* transcript levels on the relative amounts of syringyl and guaiacyl monomer subunits released by thioacidolysis of inflorescence stem lignin is consistent with earlier evidence for the contribution of COMT and CCoAOMT activities to phenolic ring methylation (Do, et al., 2007). In *myb75-1*, CCoAOMT is upregulated, while COMT is unchanged, a pattern that could have resulted in synthesis of more G subunits and less S-type lignin (Table 2.3).

In contrast to my findings, Borevitz et al. (2000) earlier reported that *MYB75* over-expression in whole Arabidopsis plants resulted in increased expression of core phenylpropanoid genes with no impact on the S/G ratio of the lignin. This discrepancy in lignin monomer ratios might be related to the differences in lignin analysis methodology (thioacidolysis *vs*. derivatization) used in the two studies. More importantly, the two studies examined very different types of tissue, with the Borevitz et al. (2000) analyses being carried out on whole plants, while my study focused on the mature inflorescence stem. Consistent with a profound developmental effect on the function of MYB75, I observed that gene expression changes induced by *MYB75* over-expression in *MYB75(o/x)* seedlings differed markedly from the patterns observed in inflorescence stems, and more closely resembled those reported for whole plants by Borevitz et al. (2000) and Tohge et. al. (2005) (Figure 2.6). *MYB111* has specificity for several genes of flavonoid biosynthesis. However, it shows specific spatial and temporal activity; controlling flavonols biosynthesis in developing seedlings, and that too primarily in cotyledons (Stracke, et al., 2007). It is clear, therefore, that the regulatory influence of transcription factors in plant tissues can be spatially and temporally conditioned.

The biosynthesis of secondary cell wall components is thought to be a highly integrated and coordinated process in which changes in the biosynthesis or regulation of an individual component can compromise the overall assembly or composition of the wall. For example, there is experimental evidence that a reduction in any secondary wall components can result in a

reduction in secondary wall thickening (Taylor, et al., 2004; Pena, et al., 2007; Zhong, et al., 1998; Zhong, et al., 2005). The increased secondary wall thickening phenotype observed in the *myb75-1* mutants could therefore be an effect associated with increased lignin deposition.

The data presented here suggests that one role of MYB75, in addition to regulating aspects of phenylpropanoid metabolism, is in more generally regulating secondary cell wall formation in the Arabidopsis stem. Loss of *MYB75* function results in the channeling of carbon toward the lignin pathway, generating increased lignin accumulation in secondary cell walls, whereas constitutive over-expression of *MYB75* leads to activation of anthocyanin biosynthesis-related genes and enhanced carbon flow into the flavonoid pathway. However, it remains possible that the visible increase in anthocyanin production (Borevitz, et al., 2000) associated with over-expression of MYB75 does not reflect its endogenous function, since over-expression of transcription factors can potentially generate artefactual pleiotropic phenotypes. Nevertheless, several MYBs have been previously shown to be involved in activation of anthocyanin biosynthesis (Gonzalez, et al., 2008), so MYB75 may be contributing to this overall regulatory activity. Consistent with such a function, transcript profiles (Table 2.5) from the *myb75-1* inflorescence stems reflect carbon flux re-distribution within the branches of phenylpropanoid metabolism, as well as into other metabolic pathways.

Chapter 3. An interaction of MYB75-KNAT7 is involved in regulation of secondary cell wall formation in the Arabidopsis inflorescence stem

3.1 SYNOPSIS

Transcription factors (TFs) function as multi-protein complexes to regulate the transcription of genes and are required for wide array of cellular functions. Secondary cell wall-associated TFs may interact among themselves to form transcriptional complexes that regulate secondary cell wall formation in Arabidopsis stem. In this chapter, a number of transcription factors with putative and known regulatory roles in secondary cell wall formation were tested for their protein-protein interactions. Many positive associations were observed and the MYB75-KNAT7 interaction was chosen for further characterization: these two TFs have been individually shown to regulate some aspects of secondary cell wall formation in Arabidopsis (Chapter 1;(Li, 2009) and, in this work, a role for their interaction in regulating secondary cell wall formation in Arabidopsis stem is examined.

3.2 INTRODUCTION

Transcriptional regulation is an important aspect of cellular functions in plants. In recent years, many transcription factors (TFs) have been predicted to be potential candidates for the regulation of secondary cell wall formation in Arabidopsis, based on gene profiling studies (Ehlting, et al., 2005; Birnbaum, et al., 2003) and protein-protein interactions (Hackbusch et al., 2005; Zimmermann et al., 2004). Some TFs were later characterized for their role in secondary cell wall biosynthesis using reverse genetics (Zhou, et al., 2009; Zhong, et al., 2008; Zhong and Ye, 2009). Cellulose, hemicellulose and lignin are the important constituents of the secondary cell wall and they are biosynthetically derived through specific biochemical pathways. However, there are many other pathways associated with, or branched from these main biosynthetic pathways. All of these require a fine tuning of regulation which is normally provided, in part, by transcription factors. In Chapter 2 of this thesis, a secondary cell wall-associated regulator MYB75, which is

also a previously characterized anthocyanin accumulation regulator (Gonzalez, et al., 2008) was shown to regulate the lignin branch of the phenylpropanoid pathway. This dual role of MYB75 in regulating divergent branches of general phenylpropanoid biosynthesis, namely lignin and anthocyanin, reflects the importance of similar TFs as potential candidates in regulating secondary cell wall formation in Arabidopsis.

Specificity of regulation by transcription factors is thought to often be provided by their modular nature, typically composed of a DNA binding domain and other effector domains. Together, these domains are responsible for both DNA and protein interactions, including the formation of multi-protein regulatory complexes, which in turn may stabilize their interaction with DNA sequence motifs in their target promoters (Wolberger, 1999). This behavior is not restricted to transcription factors; a significant percentage of all cellular proteins function in association with partner molecules or as components of large molecular assemblies (Tan, et al., 2007). Different TF interactions and, thus, different protein complexes, may change the subset of genes they can activate and thereby provide diversity in their function. Hence, to gain an accurate understanding of cellular operations, the function of proteins must be understood both in isolation and in the context of other interactive proteins. Members of many transcription factor families involved in secondary cell wall regulation have been shown to have interactions with other proteins, including members of the MYB and homeodomain families (Zimmermann, et al., 2004; Kumar, 2006). However, most of the known and potential transcription factors involved in cell wall formation have not been characterized for their physical associations via either protein-DNA or protein-protein interaction analyses.

MYBs belong to one of the largest families of plant TFs and are characterized by the conserved MYB domain, which is made up of up to three imperfect repeats called as R1, R2 and R3. Each of these repeats form a helix-turn-helix structure of about fifty three amino acids with regularly spaced tryptophan residues (Kanei-Ishii, et al., 1990; Frampton, et al., 1991; Kanei-Ishii, et al., 1997). Proteins with only an R2-R3 domain structure are more common in plants and constitute a large sub-family. These proteins are known to be involved in the regulation of phenylpropanoid or tryptophan biosynthetic pathways, control of cell fate determination, regulation of the cell cycle (Stracke, et al., 2001; Meissner, et al., 1999; Martin and Paz-Ares, 1997; Rubio, et al., 2001; Stracke, et al., 2007) as well as regulation of secondary cell wall formation (Zhong and Ye, 2007; Zhou, et al., 2009; Zhong, et al., 2008; Zhong and Ye, 2009).

Different R2R3-MYBs may bind to the promoters of target genes either alone (Patzlaff, et al., 2003b; Gomez-Maldonado, et al., 2004; Goicoechea, et al., 2005), in complex with bHLH proteins (Goff, et al., 1992; Debeaujon, et al., 2003), or with WD-40 proteins. MYB75, an R2-R3 MYB family member, has a role in regulating not only anthocyanin biosynthesis (Gonzalez, et al., 2008) but also other branches of the phenylpropanoid pathway in Arabidopsis. I showed in Chapter 2 that MYB75 also plays a role in carbon redistribution within the phenylpropanoid pathway and negatively regulates secondary cell wall formation in the Arabidopsis inflorescence stem. MYB75 also participates in protein-protein interactions with bHLH proteins and with other transcription factors (Zimmermann, et al., 2004) to form one or more anthocyanin biosynthesis regulatory complexes in Arabidopsis (Gonzalez, et al., 2008; Morita, et al., 2006).

Homeodomain transcription factors also show protein-protein interactions (Hake, et al., 2004; Bellaoui, et al., 2001; Muller, et al., 2001; Smith, et al., 2002), and KNOTTED1-like homeobox (KNOX) proteins, which form an important family of homeodomain TF's, participate in functional multi-protein complexes that regulate plant development (Hake, et al., 2004; Bellaoui, et al., 2001; Muller, et al., 2001; Hay and Tsiantis, 2010) as well as secondary cell wall formation (Brown, et al., 2005; Li, 2009). KNOX protein structure consists of the homeodomain, an ELK domain and KNOX domains that participate in physical interactions with other proteins (Burglin, 1997). Homeodomain of certain KNOX proteins interacts with other TFs, such as BELL and BEL1-like homeodoamin (BLH) proteins, to mediate protein-protein interactions and form functional complexes. The KNOX domain has small sub-domains called KNOX1 and KNOX2 which are believed to specifically interact with transcription factors like BELL proteins (Kumar, 2006). Similarly, KNOX-BLH interactions are postulated to regulate plant development (Hake, et al., 2004). Therefore, the KNOX domain seems to play an important role in the formation of functional complexes and gene regulation in Arabidopsis.

KNAT7 is a member of *KNOTTED ARABIDOPSIS THALIANA* (*KNAT*) genes and belongs to the KNOX family of homeodomain transcription factors. This TF was earlier reported to be a candidate regulator of secondary wall biosynthesis based on its co-expression with secondary cell wall biosynthetic enzymes (Persson, et al., 2005; Brown, et al., 2005; Ehlting, et al., 2005) and the *irregular xylem* (*irx*) phenotype observed in a *knat7* loss-of-function mutant. *knat7* plants have increased cell wall thickness in their interfascicular fibers (Li, 2009), suggesting a role of KNAT7 in regulating secondary wall biosynthesis. Recently it was shown

that KNAT7 physically interacts with members of the OFP family of TFs (OFP-1/OFP4) (Hackbusch, et al., 2005) and that the resulting putative complex regulates secondary cell wall formation (Li, 2009).

Arabidopsis inflorescence stems possess thick secondary cell walls in their interfascicular fibers and the regulatory role of different transcription factors in this cell wall deposition has been shown (Zhong and Ye, 2007). It is possible that these known secondary cell wall and related transcription factors may interact among themselves and/or with other proteins to form a complex and regulate secondary cell wall biosynthesis in Arabidopsis inflorescence stems. However, experimental evidence for such protein-protein interactions is still limited. Ehlting et al. (2005) proposed the involvement of many potential TFs and their complexes in regulating the secondary cell formation in inflorescence stems, including KNOX (e.g. KNAT7) and other MYB TFs. MYB75 shows an overlapping gene expression pattern in secondary cell wall-specific cell types and a loss-of-function *myb75* mutant displays thickened interfascicular fiber cell walls, similar to KNAT7. This indicates that these two transcription factors could potentially work together in this regulatory pathway. Therefore characterization of MYB75 interactions with other known and potential transcription factors involved in secondary cell wall regulation, like KNAT7, could help reveal the composition and function of TF complex(es) that may be involved in the regulation of secondary cell wall biosynthesis and lignification processes.

The work reported in this chapter reveals that both known and potential secondary cell wall-associated transcription factors can physically interact, which may indicate the formation of a functional protein complex. I established that MYB75 physically interacts with KNAT7 and identified the structural motifs underlying the MYB75-KNAT7 interaction. Additional experiments established the biological significance of this interaction in secondary cell wall formation in the Arabidopsis stem. Finally, I show that *myb75 knat7* double mutants have cell wall phenotypes similar to that of *knat7*,indicating that these two gene products are likely part of the same complex regulating inflorescence stem secondary cell wall deposition.

3.3 MATERIALS AND METHODS

3.3.1 Plant material

The Arabidopsis loss-of-function allele of MYB75 (*myb75-1*), described in Chapter 2 was used. The loss-of-function mutant of KNAT7 employed in this work was described earlier (Li, 2009), and the *myb75 knat7* double mutant was obtained by crossing *myb75-1* with *knat7-1*. Homozygous lines were selected and confirmed via PCR. Homozygous plants of each genotype were used for all experiments and compared to the relevant wild type ecotypes, WT-Col and WT-Nos (*myb75-1* is in Nossen and *knat7-1* in Columbia).

Seeds were surface-sterilized using 20% commercial bleach, cold-treated at 4°C in the dark for 2 days and plated on ½ MS agar medium (2.16 g/l MS salts, 1% sucrose, 1% Bacto-agar pH 6.0 adjusted with 1 M KOH (Murashige and Skoog, 1962). Ten-day-old seedlings were grown in pots containing a moistened Sunshine Mix #1 (Sun Gro Horticulture Canada Ltd), with a 16/8 h (day/night) and a temperature of 23°C, unless specified otherwise. For TEM, microscopy and qRT-PCR experiments, inflorescence stems were harvested from eight-week-old plants and the lower half of the stem was used for analysis. For chemical analyses, basal stem sections were dried in a 50°C oven overnight and ground in a Wiley mill to pass a 40-mesh screen.

3.3.2 Yeast two-hybrid assays

The ProQuest yeast two-hybrid system (Invitrogen) was used with full length and different fragments (for domain interaction studies) of transcription factors in pDEST32 (bait vector) or pDEST-22 (prey vector) and introduced into the yeast strain MaV203 in different combinations. Positive clones were isolated on the basis of three selectable markers: *HIS3, URA3* and *LacZ*. Positive interactions were indicated by activation of *HIS3* or *URA3*, according to the manufacturer's instructions. To compare the strength of the protein-protein interactions, a quantitative assays for β-galactosidase (β-gal) activity in liquid cultures was performed using Chlorophenol red-β-D-galactopyranoside (CPRG) as a substrate according to the in manufacturer's instructions. The different domains were amplified using specific primers (Table 3.2) and cDNA of full length MYB75 or KNAT7. These amplified fragments were then cloned in

gateway entry vector *pCR8*/GW/*TOPO*, the sequences verified and directionally recombined into the gateway destination vectors, pDEST32 and pDEST22.

3.3.3 Protoplast isolation, transfection and GUS activity assay

For protoplast isolation, leaves from 3–4 weeks old Columbia wild type plants were used and subsequent transfection and GUS activity assays were performed as described previously (Wang, et al., 2007). The plasmid DNAs for reporter and effector genes were isolated using Endofree Plasmid Maxi Kits (Qiagen, Mississauga, Ontario, Canada). Both effector plasmid (10 µg) and reporter plasmid (10 µg) were co-transfected and all assays were performed in triplicate.

3.3.4 BiFC

Gateway entry vectors with either full length MYB75 and KNAT7 or with different domains were used. For N-terminal YFP-tagged constructs, appropriate entry clone was transferred into BiFC expression vector pSAT4-DEST-nEYFP$_{1–174}$-C1 (pE3136) or pCL112 (pBATL) to produce nYFP–vectors. The same procedure was used for C-terminal YFP-tagged constructs using pSAT5-DEST-cEYFP$_{175–end}$-C1(B) (pE3130) or pCL113 (pBATL) to produce cYFP–vectors. The resulting plasmids were co-transfected into freshly prepared Arabidopsis leaf mesophyll protoplasts, and incubated for 20–22 hours (Wang, et al., 2007). YFP fluorescence was visualized using a Leica DM-6000B fluorescence microscope and photographed with a Leica digital image system (Leica Microsystems).

3.3.5 Bright field and transmission electron microscopy

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

(using an Olympus AX70 microscope) or with uranyl acetate for TEM (viewed on a Hitachi H7600 PC-TEM).

For cell wall thickness measurements, the width of the secondary cell wall in micrographs obtained from TEM was quantified in 50 cells for each genotype, using ImageJ software (http://rsbweb.nih.gov/ij/).

3.3.6 qRT-PCR

For the secondary cell wall-specific and lignin-specific gene expression study, total RNA for real-time PCR was isolated from the lower half of inflorescence stems (WT-*Col,* WT-*Nos*, *myb75-1*, *knat7-1* and *myb75 knat7*) with three biological replicates (each consisting of pooled stems from 8-10 plants) using the RNeasy Mini Protocol (Qiagen). To eliminate residual genomic DNA, the RNA was treated with RNAse-free DNAseI according to the manufacturer's instructions (Qiagen). The concentration of RNA was measured using the absorbance at 260 nm and the quality was assessed using the A260/A280 ratio. Total RNA (2 µg) was reverse transcribed using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. cDNA was diluted (1:20) and 2 µl was used in each reaction in a 20 µl reaction volume. PCR amplification was performed with gene-specific primers for secondary cell wall or lignin-specific genes (Table 3.1), using *Actin8* as a normalization control (Table 3.1). *Actin8* was chosen as the reference gene for this study as its expression has been shown to be relatively invariant (An, et al., 1996). However, in my experiments, stable expression of *Actin8* was reconfirmed in inflorescence stem samples (data not shown). The cDNA was amplified using the PerfeCta™ qPCR FastMix (Quanta Biosciences) on the DNA Engine Opticon® 2 (Bio-Rad). Differences in gene expression, expressed as fold-change relative to control, were calculated using the [delta][delta]$C_t = 2^{[\text{delta}]Ct,\text{Actin} - [\text{delta}]Ct,\text{gene}}$ method. Each measurement was carried out in triplicate, and the error bars represent standard error of the mean (SEM) of fold-changes for the three biological replicates.

3.3.7 Primers

Table 3.1 Primers used in this study (Chapter 3).

Gene ID	Name	Primer sequence (5'-3')
At2g37040	PAL1 (q-RT)	PAL1-L: AAGATTGGAGCTTTCGAGGA
		PAL1-R: TCTGTTCCAAGCTCTTCCCT
At2g30490	C4H (q-RT)	C4H-L: ACTGGCTTCAAGTCGGAGAT
		C4H-R: ACACGACGTTTCTCGTTCTG
At1g51680	4CL1 (q-RT)	4CL1-L: TCAACCCGGTGAGATTTGTA
		4CL1-R: TCGTCATCGATCAATCCAAT
At5g48930	HCT (q-RT)	HCT-L: GCCTGCACCAAGTATGAAGA
		HCT-R: GACAGTGTTCCCATCCTCCT
At2g40890	C3H1 (q-RT)	C3H1-L: GTTGGACTTGACCGGATCTT
		C3H1-R: ATTAGAGGCGTTGGAGGATG
At4g34050	CCOMT 1 (q-RT)	CCOMT1-L: CTCAGGGAAGTGACAGCAAA
		CCOMT1-R: GTGGCGAGAAGAGAGTAGCC
At1g15950	CCR1 (q-RT)	CCR1-L: GTGCAAAGCAGATCTTCAGG
		CCR1-R: GCCGCAGCATTAATTACAAA
At4g36220	F5H (q-RT)	F5H-L: CTTCAACGTAGCGGATTTCA
		F5H-R: AGATCATTACGGGCCTTCAC
At5g54160	COMT1 (q-RT)	COMT1-L: TTCCATTGCTGCTCTTTGTC
		COMT1-R: CATGGTGATTGTGGAATGGT
At4g34230	CAD5 (q-RT)	CAD5-L: TTGGCTGATTCGTTGGATTA
		CAD5-R: ATCACTTTCCTCCCAAGCAT
At4g32410	CesA1 (q-RT)	CesA1-L: GGTATTTATTGCGGCAACCT
		CesA1-R: ATCCAACCAATCTCTTTGCC
At5g05170	CesA3 (q-RT)	CesA3-L: ACAGCCAACACAGTGCTCTC
		CesA3-R: TGGTACCCATTTACGAGCAA
At2g21770	CesA6 (q-RT)	CesA6-L: TGCCCTTGAGCACATAGAAG
		CesA6-R: GCACTCCACCATTTAGCAGA
At5g44030	CesA4 (q-RT)	CesA4-L: GGATCAGCTCCGATCAATTT
		CesA4-R: ACCACAAAGGACAATGACGA
At5g17420	CesA7 (q-RT)	CesA7-L: CAGGCGTACTCACAAATGCT
		CesA7-R: TGTCAATGCCATCAAACCTT
At4g18780	CesA8 (q-RT)	CesA8-L: ACGGAGAGTTCTTTGTGGCT
		CesA8-R: GGTCTGTGTTGGAACAATGG
At5g54690	IRX8 (q-RT)	IRX8-L: GTGGTCACAGGGAAAGGATT
		IRX8-R: AGCAAGAGAGGAGCAAGGAG
At2g37090	IRX9 (q-RT)	IRX9-L: TTTGCGGGACTAAACAACAT
		IRX9-R: ATCGGAGGCTTTGTCTCTGT
At2g28110	FRA8 (q-RT)	FRA8-L: GACTTGTTGAATCGGTGGCTC
		FRA8-R: GAAAGAGTTTGACCTTCTAAC
At1g56650	MYB75 (q-RT)	MYB75-L: TTCTTCGCCTTCATAGGCTT
		MYB75-R: AGGAATGGGCGTAATGTCTC
At1g62990	KNAT7 (q-RT)	KNAT7-L: AAATTGGTGGAGGAGACAGG
		KNAT7-R: TCCTCTTGCGTTGGTTAATG
At1G49240	ACT8	Act8-L: TCTAAGGAGGAGCAGGTTTGA
		Act8-R: TTATCCGAGTTTGAAGAGGCTAC
At1g62990	KNAT7-FL	KNAT7-F: ATGCAAGAAGCGGCACTAG
		KNAT7-R: TTAGTGTTTGCGCTTGGACTT
	KNAT7-KNOX1	KNAT7-KNOX1-F: ATGCGTTCTTACGCTTCCACG
	KNAT7-KNOX2	KNAT7-KNOX2-F: ATGCGTTCTTACGCTTCCACG
		KNAT7-KNOX2-R: TTACCCTTCTCCTAAAGTTGC
	KNAT7-ELK+HM	KNAT7-HM-F: ATGGAAAGAGTCAGACAAGAA
At1g56650	MYB75-FL	MYB75-F: ATGGAGGGTTCGTCCAAAG
		MYB75-R: CTAATCAAATTTCACAGTCTCTCCATC
	MYB75-R2	MYB75 R2-F: ATGTCCAAAGGGCTGCG
		MYB75 R2-R: TTATTCAAATAGTTCAACCATCTTAAT
	MYB75-R3	MYB75 R3-F: ATGAAGCCAAGTATCAAGAGAGG
		MYB75 R3-R: TTAATGTTTCTTACTCAGATGAGTGTT
	MYB75-R2-R3	MYB75 R2-R3- F: ATGGAGGGTTCGTCCAAAG
		MYB75 R2-R3- R: TTACGGTTCATGTTTCTTACTCAGAT

3.3.8 Accession numbers

GenBank database accession numbers for the genes studied in this work and not indicated elsewhere are *MYB75* (At1g56650), *C4H* (At2g30490), *PAL1* (At2g37040), *4CL1* (At1g51680), *C3H1* (At2g40890), *HCT* (At5g48930), *F5H1* (At4g36220), *CCoAOMT1* (At4g34050), *COMT* (At5g54160), *CCR1* (At1g15950), *CAD5* (At4g34230), *CesA4* (At5g44030), *CesA7* (At5g17420), *CesA8* (At4g18780), *IRX8* (At5g54690), *IRX9* (At2g37090), *CesA1* (At4g32410), *CesA3*(At5g05170), *CesA6* (At5g64740), *FRA8* (At2g28110).

3.3.9 Chemical analysis

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

3.4 RESULTS

3.4.1 Transcription factors known or predicted to be involved in secondary cell wall regulation show protein-protein interactions

In order to systematically investigate potential interactions among secondary cell wall regulating TFs, I focused on some of the known and potential factors as indicated (Table 3.2) and assayed these for protein-protein interactions in a yeast two-hybrid (Y2H) all-against-all matrix (Figure 3.1). All the tested candidates were combined with the GAL4 DNA binding domain (BD) and GAL4 activation domain (AD) and assayed for their ability to bind themselves or to each other

using a Proquest Y2H kit (Invitrogen). The wild type yeast strain was transformed with the BD- or AD- construct. Interaction was studied using appropriate reporter genes. Yeast growth on SC-Leu-His in the presence of at least 25 μM 3AT above the background and growth on SC-Leu-Ura plates was considered a positive interaction result. MYB, TT8 and bHLH012 show inherent transcription activating function constructs when used as binding domain fusions in the yeast two-hybrid system and so it was not possible to use them as BD constructs.

Table 3.2 Known and potential secondary cell wall regulators in Arabidopsis tested in yeast two-hybrid assay

AGI Code	Gene Family	Name	Role in secondary cell wall formation	Source	Reference
At1g56650	MYB	MYB75/ PAP1	Known	Secondary cell wall-associated pathway Protein-protein interaction	Chapter1; Borevitz et al., 2000; Gonzalez et al., 2008; Zimmermann et al., 2004
At1g66230	MYB	MYB20	Known	Microarray, Reverse genetics	Ehlting et al., 2005; Zhong et al., 2008
At1g79180	MYB	MYB63	Known	Microarray, Reverse genetics	Ehlting et al., 2005; Zhong et al., 2008
At5g16610	MYB	MYB43	Known	Microarray, Reverse genetics	Ehlting et al., 2005; Zhong et al., 2008
At3g13540	MYB	MYB5	Potential	Secondary cell wall-associated pathway, Protein-protein interaction	Gonzalez et al., 2009; Li et al., 2009
At1g62990	HD	KNAT7	Known	Microarray, Reverse genetics	Brown et al., 2005; Ehlting et al., 2005; Li, E., 2009
At5g24800	bZIP	bZIP9	Potential	Microarray	Ehlting et al., 2005
At5g65210	bZIP	bZIP47	Potential	Microarray	Ehlting et al., 2005
At1g32770	NAC	SND1	Known	Reverse genetics	Zhong et al., 2006
At5g42200	C3H	No name	Potential	Microarray	Ehlting et al., 2005
At5g07580	AP2-EREBP	No name	Potential	Microarray	Ehlting et al., 2005
At1g29950	bHLH	bHLH144	Potential	Microarray	Ehlting et al., 2005
At4g09820	bHLH	TT8	Potential	Secondary cell wall-associated pathway, Protein-protein interaction	Nesi et al., 2000; Zimmermann et al., 2000
At4g29100	bHLH	bHLH068	Potential	Microarray	Ehlting et al., 2005
At4g00480	bHLH	bHLH012/ MYC1	Potential	Protein-protein interaction	Zimmermann et al., 2000

	AD-KNAT7	AD-bHLH144	AD-bZIP9	AD-SND1	AD-C3H	AD-AP2	AD-MYB43	AD-bHLH068	AD-bZIP47	AD-MYB63	AD-MYB20	AD-MYB5	AD-MYB75	AD-DST22
BD-KNAT7	-	-	-	-	-	-	-	-	-	-	-	+	+	-
BD-C3H	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BD-AP2	-	-	-	-	-	-	-	-	+	-	-	-	-	-
BD-bZIP47	-	-	-	-	-	+	-	-	-	-	-	-	-	-
BD-TT8	-	-	-	-	-	-	-	-	-	+	-	+	+	-
BD-bHLH012	-	-	-	-	-	-	-	-	-	-	-	-	+	-
BD-DST32	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 3.1 *In vitro* protein–protein interactions among potential secondary cell wall-associated transcription factors as determined by yeast two-hybrid assay. Known interactions (shown in dark grey box, Zimmermann et. al 2004) were used as a positive control and empty vectors were co-transformed to be used as a negative control

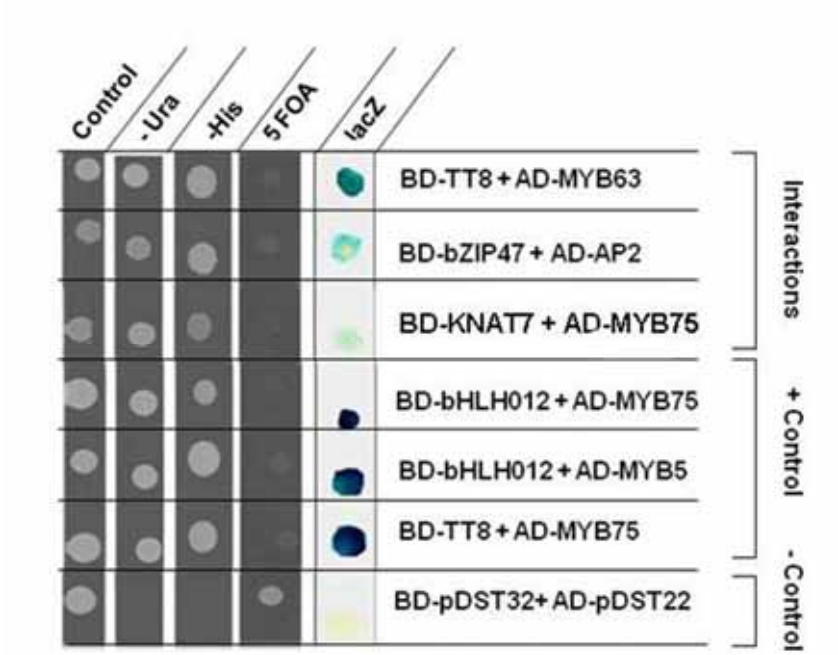


Figure 3.2 Confirmation of yeast two-hybrid interactions by different reporter genes. Positive clones were isolated on the basis of three selectable markers: *HIS3, URA3 LacZ* and 5-FOA. Control means cells grown on SC-Leu-Trp media. 5-FOA is 5-Fluoroorotic acid.

Within this assay matrix, positive yeast 2-hybrid interactions were observed between MYB75 and KNAT7, AP2 and bZIP47, KNAT7 and MYB5 and between TT8 and MYB63

(Figure 3.1). MYB75 and TT8 have previously been shown to interact (Zimmermann, et al., 2004), and this interaction therefore served as a positive control for these assays.

All positive interactions detected were subsequently confirmed using different reporter genes (Figure 3.2) and the strength of the interactions was assayed using a chlorophenol red- β -D-galactopyranoside (CPRG) colorimetric reporter (Figure 3.3).

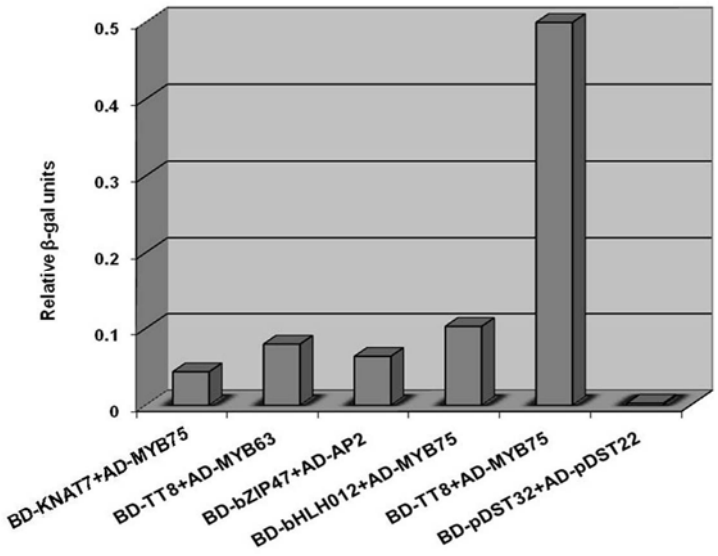


Figure 3.3 Quantification of strength of yeast two-hybrid interactions by the CPRG assay. The MYB75-TT8 and MYB75-bHLH012 interactions were used as positive controls (Zimmermann et. al 2004). Empty vectors pDST32 and pDST22 were used as negative controls.

3.4.2 MYB75 and KNAT7 show interaction *in vivo*

MYB75 (Chapter 2) and KNAT7 (Li, 2009) were shown to be negative regulators of secondary cell wall formation in the Arabidopsis inflorescence stem. To understand the biological role of these two functionally similar TFs as a part of a potential complex, the MYB75-KNAT7 interaction was chosen for detailed study in this work. The bi-molecular florescence complementation assay (BiFC) using a split yellow florescent protein (YFP) was used to demonstrate that the MYB75-KNAT7 interaction could also be observed *in vivo* in Arabidopsis protoplasts (Figure 3.4). MYB75 was fused to N-terminal split YFP and KNAT7 to C-Terminal fragment of the fluorescent protein, neither of which is capable of fluorescence alone. Transcription factors (MYB75 and TT8) were fused to different fragments of YFP and this

interaction was used as a positive control in this experiment based on their known interaction in yeast (Zimmermann, et al., 2004)(Figure 3.4). This interaction was confirmed *in vivo* but did not show nuclear localization as expected for these two known transcription factors. Using the Arabidopsis leaf mesophyll protoplast transient expression system (Tiwari, et al., 2006), I transformed different combinations of fusion constructs. Reconstitution of complete YFP as seen by the fluorescence was visible in YFP-C-KNAT7 with YFP-N-MYB75 combination and in the positive control (TT8-MYB75 interaction (Figure 3.4). The MYB75-KNAT7 interaction appears to be both nuclear localized as well as in cytoplasm in the transformed protoplasts. Co-transformed YFP-N-MYB75 and YFP-C-EV (EV, empty vector) constructs, as well as YFP-C-KNAT7 and YFP-N-EV constructs, were used as negative controls.

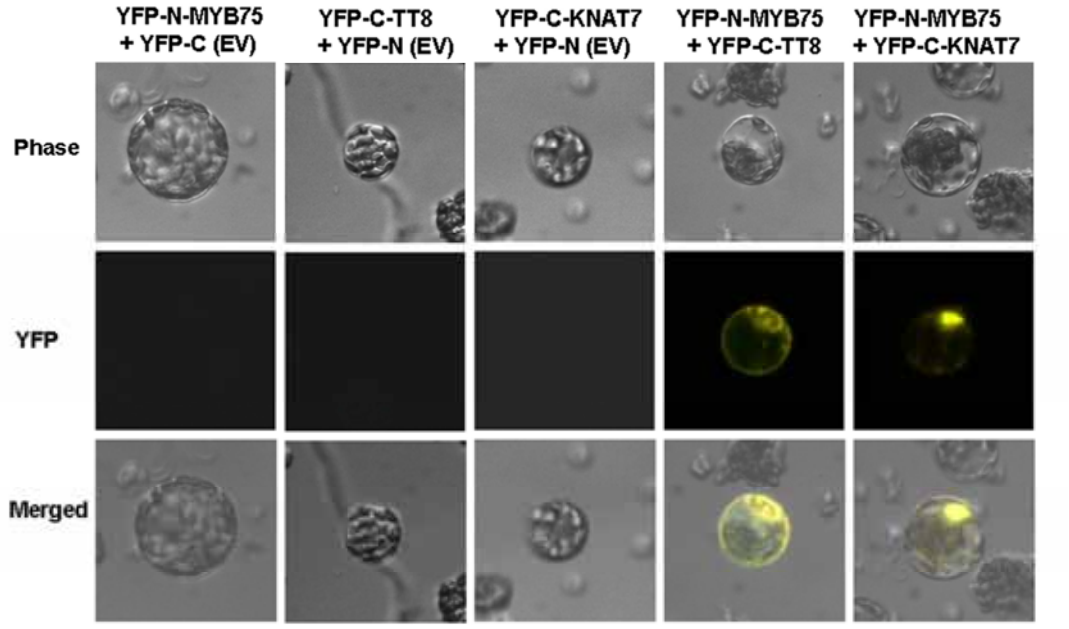


Figure 3.4 Confirmation of yeast two-hybrid interactions by BiFC assay in Arabidopsis protoplasts. The MYB75-TT8 interaction was used as a positive control (Zimmermann et. al., 2004).

Further, the MYB75-KNAT7 interaction was confirmed *in vivo* by using a protoplast transfection assay for transcription activation/repression activity (Wang, et al., 2007)(Figure 3.5). In this system, a β-glucuronidase (*GUS*) gene expressed under the control of the Gal4 DNA binding site (*Gal4*: *Gus*) was used as a reporter plasmid and was co-transfected with effector plasmids (Figure 3.5A). The first effector plasmid employed was a plasmid encoding only the

yeast Gal4 DNA binding domain (GD), which resulted in a low level of the GUS reporter gene expression, as measured by GUS enzyme activity (left bar, Figure 3.5B) This served as the control level of GUS activity in this experiment. When the MYB75 was fused with GD and used as an effector plasmid co-transfected with the reporter, a slight increase in GUS activity was seen, indicating that MYB75 may act as a weak transcriptional activator (middle bar, Figure 3.5B). However, when the effector plasmid encoding hemagglutinin tagged (HA) KNAT7 protein was co-transfected along with the GD-MYB75 and reporter plasmids, suppression of the GUS activity was observed, even though the HA-KNAT7 construct alone had no ability to bind to the promoter of the GUS reporter (right bar, Figure 3.5B). This confirmed the physical interaction between MYB75 and KNAT7 and also confirmed the repressor activity of KNAT7 (Li, 2009).

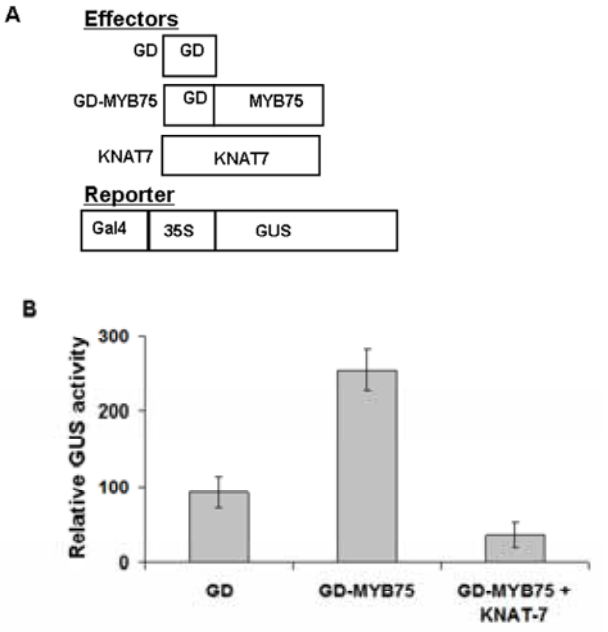


Figure 3.5 MYB75-KNAT7 interaction confirmed by protoplast transfection assay. (A) Schematic diagram of the effector and reporter constructs used in the transfection assays. Effector genes were either fused to the *Ga14* DNA-binding domain (GD) in-frame with MYB75, no fusion of the GD and KNAT7 fused with Hemagglutinin epitope (HA) tag. Effector gene plasmid DNA and reporter gene *Gal4: GUS* plasmid DNA were co-transfected into Arabidopsis leaf mesophyll protoplasts. The transfected protoplasts were incubated in darkness for 20–22 h and GUS activity was assayed after. Shown are means ± SE of three replicates. MYB75 and KNAT7 show interaction as seen by repression in the GUS activity.

3.4.3 The MYB75-KNAT7 interaction is based on the R2-R3 repeat of the MYB75 domain and KNOX2 domain of KNAT7

MYB75 belongs to R2R3 family of MYB transcription factors and is composed of two adjacent imperfect repeats, each with about 52 amino acid residues that adopt a helix-turn-helix conformation in addition to an activation domain. The activation domain of MYB75/PAP1 has been determined to the 58 C-terminal residues of the protein (Zimmermann, et al., 2004). To identify the regions required for the interaction of MYB75 with the KNAT7, separate domain regions of MYB75 were analyzed (Figure 3.6A) using the yeast two-hybrid system. MYB75 protein fragments were cloned and fused to the GAL4-AD and tested for interaction with the full length KNAT7 (FL-KNAT7) transcription factor in BD. Positive interaction was identified between R3 domain and FL-KNAT7 as evident from the growth of the transformant on SC-Leu-His + 25mM 3AT and on SC-Leu-Ura selection media (Figure 3.6B). I was not able to clone the activation domain, so it was not tested in this experiment. FL-KNAT7 and full length MYB75 co-transformation was used as a positive control. Appropriate negative controls (empty vectors) were used. No growth was seen in co-transformation of FL-KNAT7 with other domains of MYB75 (Figure 3.6B).

To further confirm this interaction *in vivo*, I used BiFC and the Arabidopsis protoplast system. Positive fluorescence was detected in protoplasts co-transfected with plasmids encoding the MYB75 R3 domain and FL-KNAT7 as well as those encoding the MYB75 R2-R3 domain and FL-KNAT7. These yeast two-hybrid results indicate that the R3 domain is necessary for the interaction with MYB75 (Figure 3.6C). KNAT7 is a homeodomain transcription factor and belongs to KNOX class of this family. All plant KNOX proteins have an ELK domain and a KNOX (MEINOX) domain. The MEINOX domain consists of two smaller sub-domains, KNOX1 and KNOX2. To identify which regions of KNAT7 are involved in the interaction of KNAT7 with MYB75, separate domains of KNAT7 were subcloned, converted to Y2H constructs (Figure 3.7A) and then tested with identified interaction domains of MYB75 (R3 and R2-R3) (Figure 3.6A). The interaction between full length KNAT7 and FL-MYB75 was used as a positive control. KNAT7 homeodomain and the KNOX1 (K1) or KNOX1-KNOX2 (K1-K2) did not show any positive interactions with MYB75 R2R3 or R3 domains in the yeast two-hybrid assays (data not shown). However, a weak interaction (growth of co-transformants only on SC-

Leu-His+3AT and not on stringent selection marker, SC-Leu-Ura) was observed between the MYB75 R2R3 domain and the K2 domain of KNAT7 (Figure 3.7B).

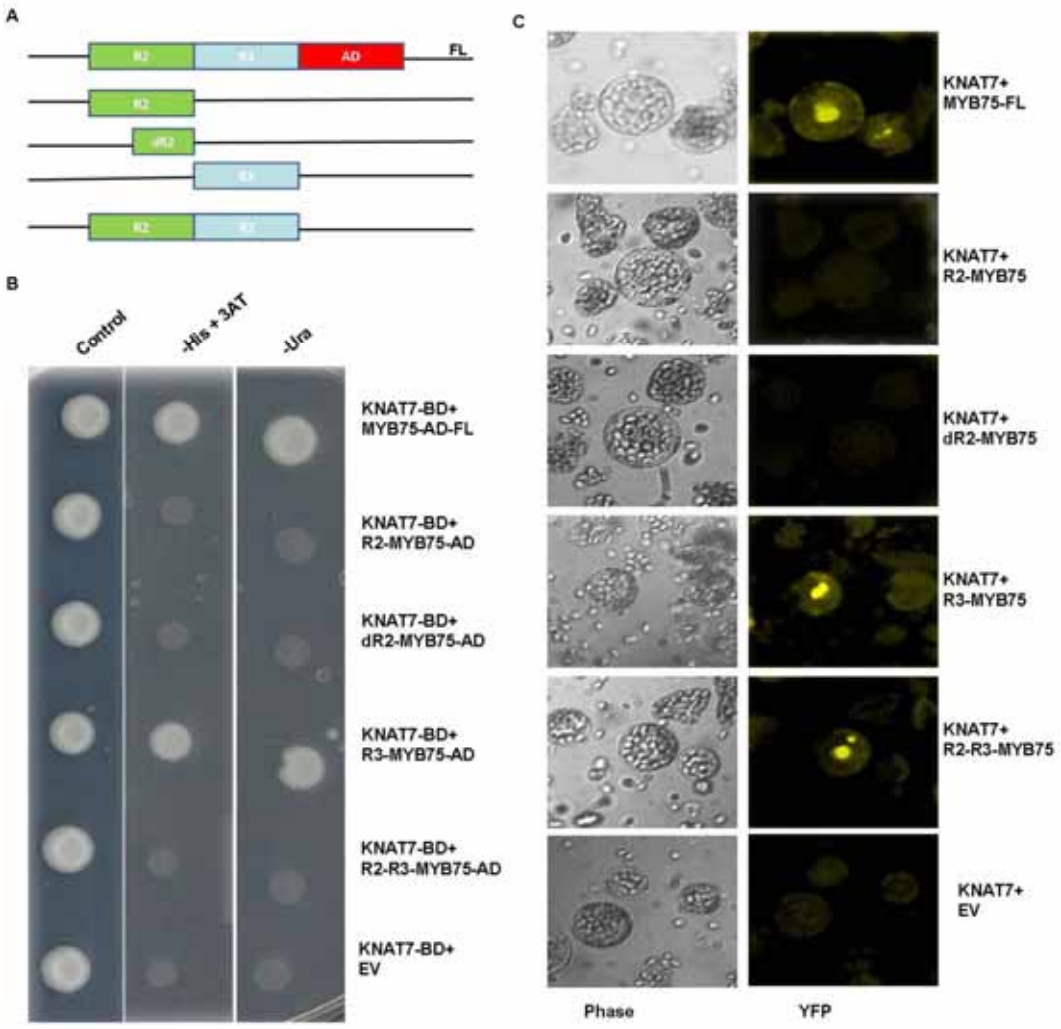


Figure 3.6 Identification of MYB75 domain(s) involved in protein-protein interaction with KNAT7. (A) Different domains of MYB75 used to test the interaction with Full length (FL) KNAT protein using yeast two-hybrid and BiFC assays (colored boxes). (B Yeast two-hybrid assay to test the interaction. Different domains of the MYB75 protein were fused to the GAL4 activation domain (AD) in yeast-two hybrid assays and tested for interaction with KNAT7-full length (Figure 3.7A) protein fused to the DNA-binding domain (BD). Yeast growth on SC-LeU-His+3AT and SC-Leu-Ura served as a positive control interaction. (C) BiFC assays using split YFP to test the same interactions *in vivo* using Arabidopsis protoplast system.

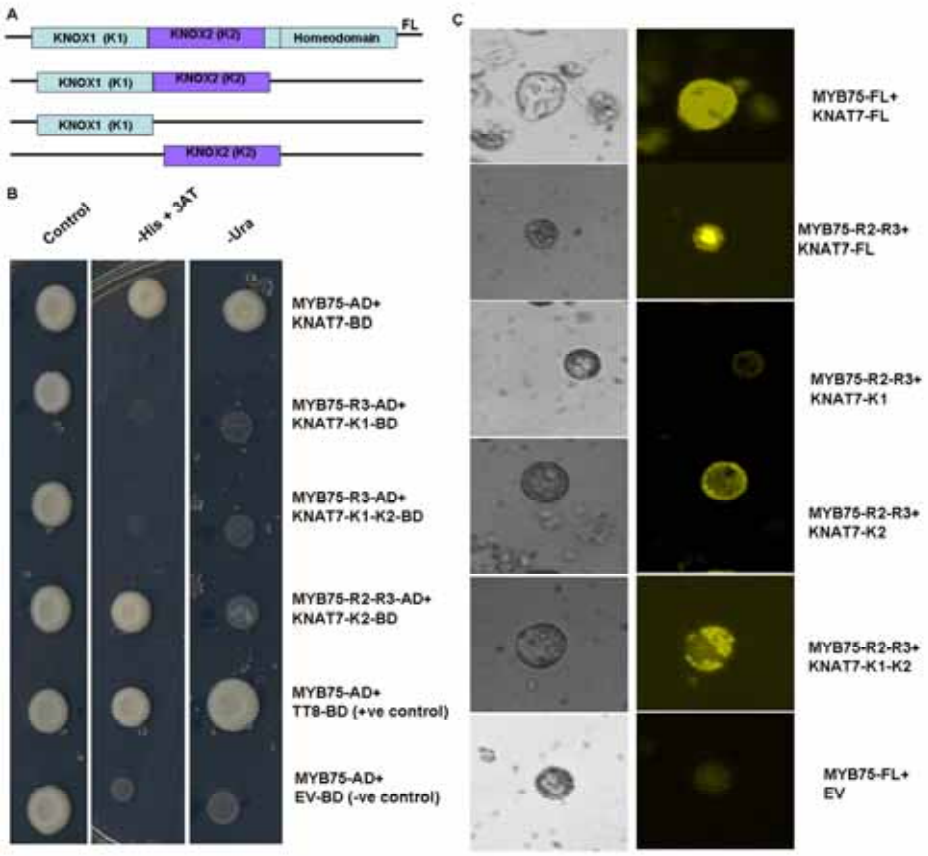


Figure 3.7 Identification of KNAT7 domain(s) involved in protein-protein interaction with MYB75. (A) Different domains of KNAT7 were used to test the interaction with the MYB75 R2-R3 domain (Figure 3.6 A) using yeast two-hybrid and BiFC assay (colored boxes). (B)Yeast two-hybrid assay to test the interaction. Different domains of MYB75 (Figure 3.7A) proteins were fused to the GAL4 activation domain (AD) in yeast-two hybrid assays and were tested for interaction in yeast with different domains of KNAT7 protein fused to DNA binding domain (BD). Yeast growth on SC-LeU-His+3AT and SC-Leu-Ura served as a positive control interaction. (C) BiFC using split YFP was used to test the same interactions *in vivo* using the Arabidopsis protoplast system.

These interactions were also tested *in vivo* using the BiFC and the protoplast system. YFP fluorescence was observed when the R2-R3 domain of MYB75 was co-transfected either with the BiFC plasmid encoding the KNAT7-K2 or the KNAT7-K2-K3 domains (Figure 3.7C). No interaction was observed between the KNAT7-K1 domain and the MYB75-R2-R3 domain. No interaction was detected when different KNAT7 domains were tested alone with the R3 domain of MYB75 (Figure 3.7C). These domain interaction results indicate that the physical interaction between KNAT7 and MYB75 is dependent primarily on the R2-R3 domain of MYB75 and the

KNOX2 domain of KNAT7. It is notable in Figure 3.7 that most of the interactions appear to be outside the nucleus.

3.4.4 MYB75 and KNAT7 function in the same pathway to regulate secondary cell wall formation in the Arabidopsis stem

To test the combined roles of MYB75 and KNAT7 in regulating secondary wall formation, I generated double mutants by crossing a *myb75-1* mutant line (Chapter 2)(Teng, et al., 2005) with *knat7-1* (Li, 2009). Double mutants were identified in the F2 population by PCR-aided genotyping. Homozygous double mutant plants showed no morphological differences compared with wild type (data not shown).

Light microscopic analysis of cross-sections from the basal part of inflorescence stems of double mutants revealed that *knat7 myb75* plants exhibited similar phenotypes to *knat7-1* (Brown, et al., 2005; Li, 2009), with collapsed vessels and increased thickness of interfascicular fiber cell walls (Figure 3.8A). Higher resolution examination by transmission electron microscopy (TEM) in single and double mutant (*myb75-1, knat7-1* and *myb75 knat7*) inflorescence stems compared to the *WT* confirmed the presence of thickened cell walls in interfascicular fibers, and again showed that the double mutant phenotype most closely resembled that of the *knat7-1* mutant (Figure 3.8B). The electron micrographs were used to quantify the thickness of the cell wall, vessels and xylary fibers and these measurements confirmed that the double mutant phenotype was similar to *knat7-1* phenotype, in terms of the wall thickness of interfascicular fiber cells and vessels (Figure 3.8C).

Since changes in interfascicular fiber cell wall thickness in the double mutant interfascicular fibers could possibly be accompanied by changes in the secondary cell wall constituents, I compared the cell wall chemistry of the WT with the single and double mutants. This analysis revealed that the Klason lignin content was significantly greater in lower inflorescence stems of *knat7-1*, *myb75-1* and *myb75 knat7* plants relative to WT (both Columbia and Nossen)(Figure 3.9). Total sugar analysis performed on the lower stem sections in *myb75 knat7* plants showed a decrease in total glucose content (Figure 3.10), and also decreased levels of arabinose and mannose relative to both wild types (Col and Nos).

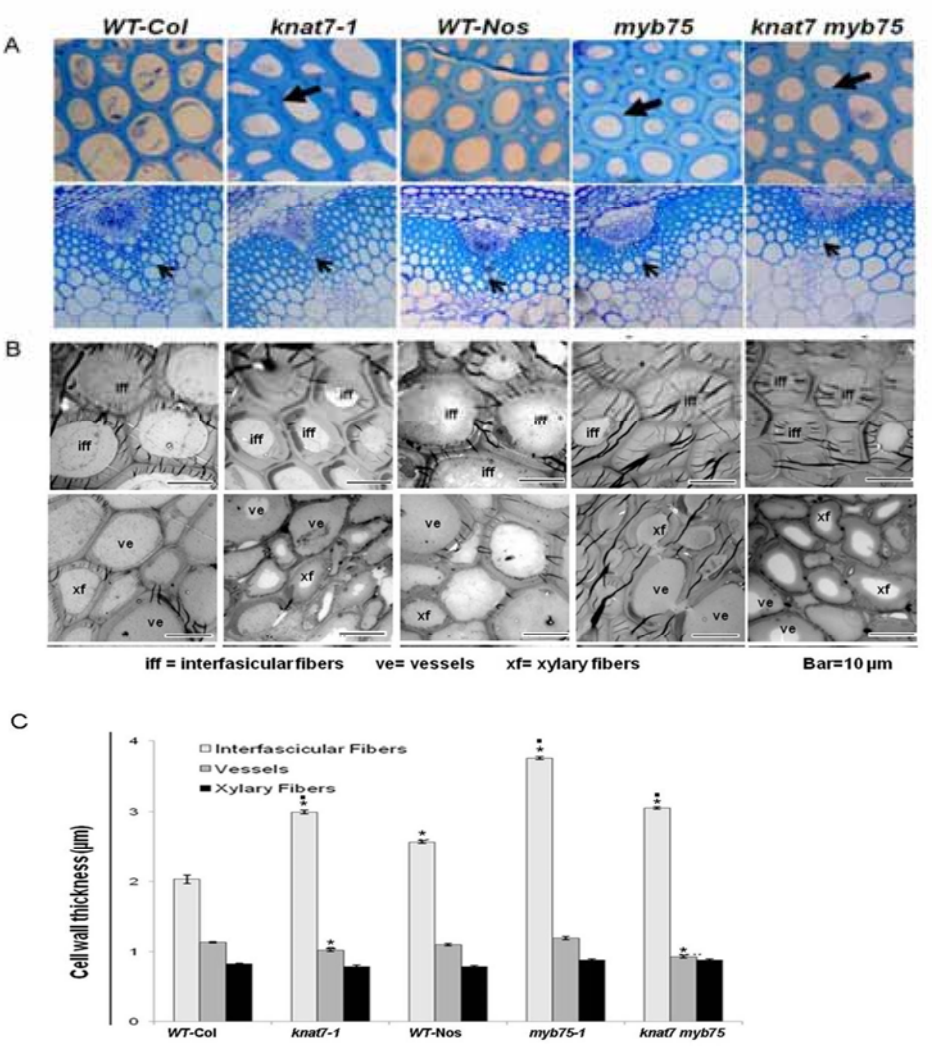


Figure 3.8 Secondary wall thickening in fibers in *myb75-1, knat7-1* and *myb75 knat7* plants. The bottom portion of the Arabidopsis inflorescence stem was used for assessment of secondary walls in fibers and vessels. (A) Toluidine Blue staining of stem cross-sections in mutants and *WT* (Top). Higher magnification of the Toluidine Blue-stained sections (Bottom). *myb75-1* (Chapter 1), *knat7*-1 (Li, E. (2009) and double mutants *(myb75 knat7)* show thickened interfascicular fiber walls (indicated by black arrows), while *knat7-1* (Brown et al., 2005) and *myb75 knat7* have a collapsed xylem phenotype (B) Transmission electron microscopy of wild type, *myb75-1*,*knat7-1* and double mutant *(myb75 knat7)* stem sections. (C) Measurements of secondary cell wall thickness in *WT* and mutants (as indicated) (µm)). The wall thickness was measured from transmission electron micrographs of fibers and vessels. Data are means (µm) ± SE from 50 cells. **if**, interfascicular fiber; **ve**, vessel; **xf**, xylary fiber. Bars = 100 µm for toludine blue stained sections (upper panel and 50 µm (lower panel indicated) in (A), and 10 µm in (B). *myb75-1* show thickened cell wall as compared to *WT*. ▪ indicates significant difference from WT-Nos. * indicates significant difference from WT-Col. *myb75-1* is in Nos background and so is compared to WT-Nos and knat7-1 is in Col background and so compared to WT-Col.

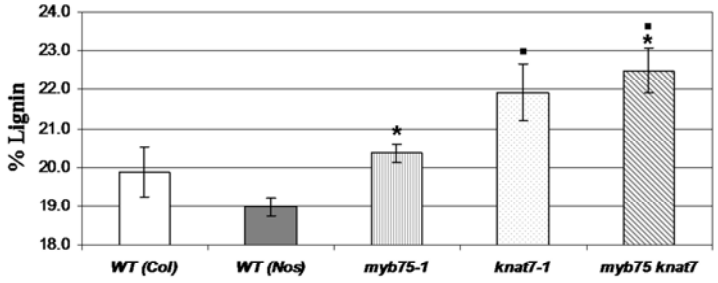


Figure 3.9 Lignin content in the inflorescence stems of the wild type and mutants as determined by Klason analysis. The analysis reveals a higher lignin content in *myb75-1* as compared to *WT*. Each data point is the mean (mg lignin/100 mg dry cell walls) ± SE of two assays. * indicates significant difference from WT-Nos. ▪ indicates significant difference from WT-Col. *myb75-1* is in Nos background and so is compared to WT-Nos and knat7-1 is in Col background and so compared to WT-Col.

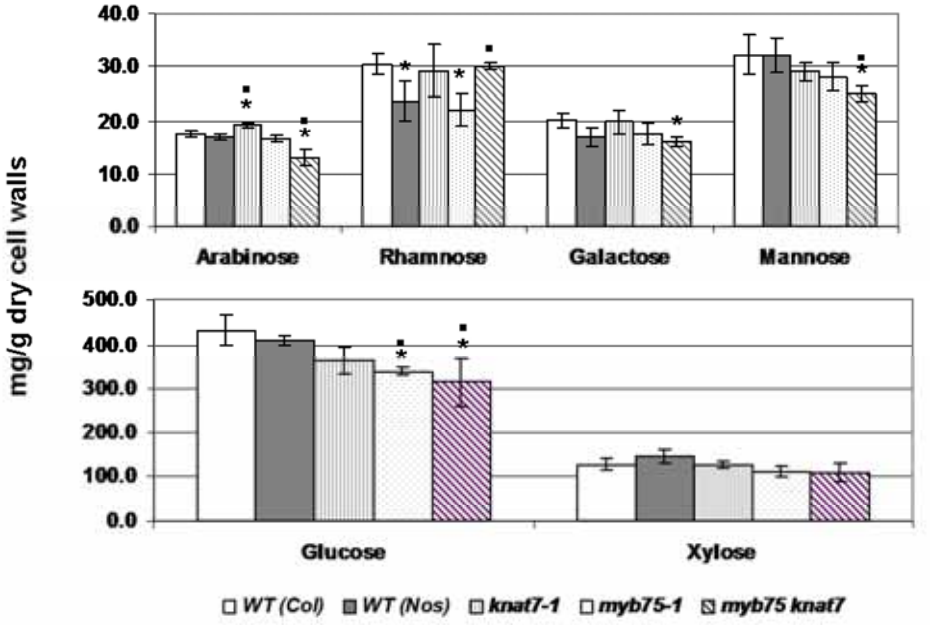


Figure 3.10 Carbohydrate content in the lower stems of the wild type and mutants as determined by Klason analysis. Each data point is the mean (mg/g dry cell walls) ± SE of two separate assays with three replicates in each assay. * indicates significant difference from WT-Col. ▪ indicates significant difference from WT-Nos. *myb75-1* is in Nos background and so is compared to WT-Nos and knat7-1 is in Col background and so compared to WT-Col.

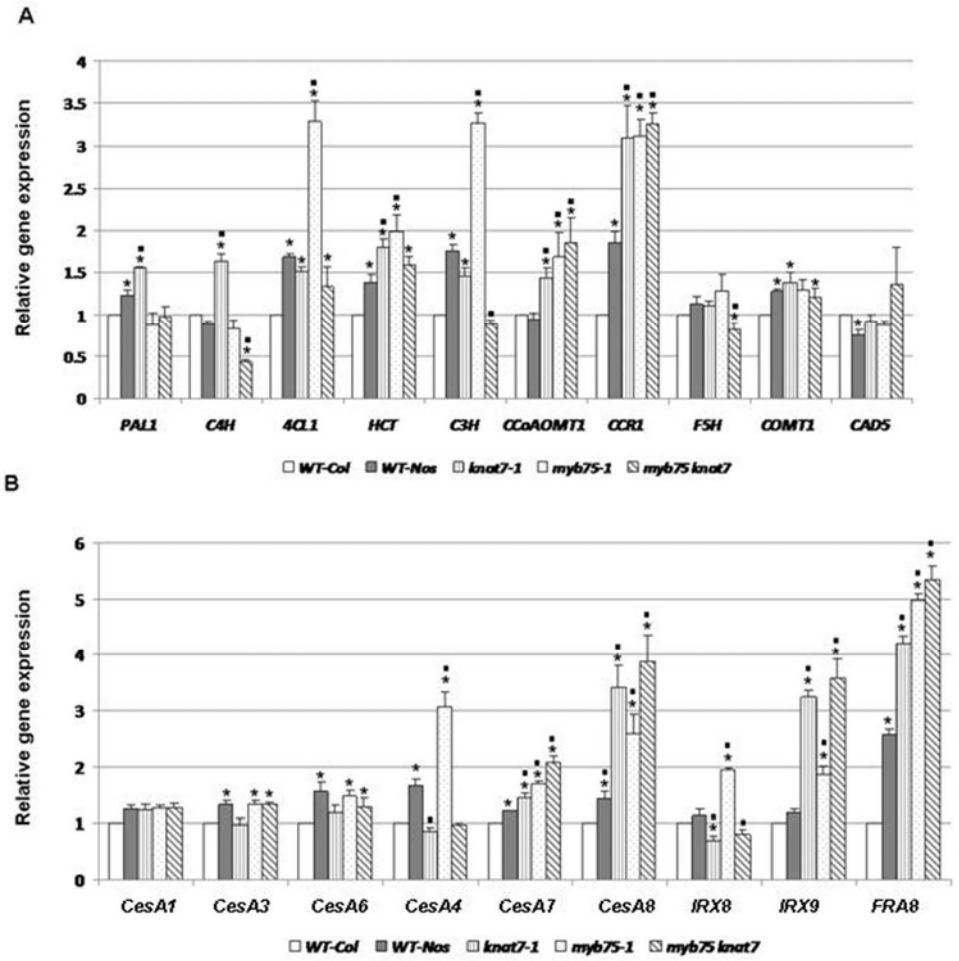


Figure 3.11 Secondary cell wall-associated gene expression in lower stems of WT-Nos, *myb75-1, knat7-1* and double mutant (*myb75 knat7*) relative to WT-Col plants. (A) Expression of lignin biosynthetic genes in lower inflorescence stems of *MYB75* and *KNAT7* loss-of-function and double loss-of function (*myb75 knat7*) mutants and *WT-Nos* compared with the wild type-Columbia by real-time quantitative PCR analysis. The expression levels of most of the genes in the lignin pathway were examined. PAL1, phenylalanine ammonia lyase 1; C4H, cinnamate 4-hydroxylase; 4CL1, 4-coumarate CoA ligase 1; HCT, hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyltransferase; C3H1, coumarate 3-hydroxylase 1; CCoAOMT1, caffeoyl CoA 3-*O*-methyltransferase 1; CCR1, cinnamoyl CoA reductase 1; F5H1, ferulate 5-hydroxylase 1; COMT1, caffeic acid *O*-methyltransferase 1; CAD5, cinnamyl alcohol dehydrogenase 5. (B) Expression of secondary wall biosynthetic genes in lower inflorescence stems of *MYB75* and *KNAT7* loss-of-function and double loss-of function (*myb75 knat7*) mutants and *WT-Nos* compared with the wild type-Columbia and real-time quantitative PCR analysis. The expression levels of genes involved in the formation of cellulose in the primary cell wall (*CesA1, CesA3, CesA6*), cellulose in the secondary cell wall (*CesA4*, *CesA7* and *CesA8*) and xylan (*FRA8, IRX8 and IRX9*), were examined. Error bars represent SE of three replicates. ▪ indicates significant difference from WT-Nos. * indicates significant difference from WT-Col. *myb75-1* is in Nos background and so is compared to WT-Nos and knat7-1 is in Col background and so compared to WT-Col.

To test further the roles of both KNAT7 and MYB75 in secondary cell wall formation, I performed real-time PCR analysis for genes encoding enzymes that are involved in secondary cell wall biosynthesis (lignin-, cellulose- and hemicellulose-associated gens). Real time PCR analysis was performed on *myb75-1*, *knat7-1*, *myb75 knat7* double mutant and WT (Nos) and was compared to WT (Col) gene expression. Many of the lignin-specific genes examined were found to be up-regulated in the *myb75-1, knat-7* and *myb75 knat7* stems (Figure 3.11A). Similar to earlier observations for the *myb75-1* mutants (Chapter 2), *knat7-1* and *myb75-1 knat7-1* primary cell wall-associated cellulose synthase genes (*CesA3, CesA5* and *CesA6*) did not show any change in expression, whereas genes encoding the cellulose synthase isoforms (CesA4, 7 and 8) believed to be specifically responsible for biosynthesis of secondary cell wall cellulose microfibrils (Taylor, et al., 2004) showed upregulation (Figure 3.11 B). Expression of xylan biosynthesis genes, *IRX8*, *IRX9* and *FRA8* (Persson, et al., 2007; Pena, et al., 2007) was increased in the *myb75-1, knat7-1* and double mutant genotypes. Comparison of gene expression profiles in the double mutant with those of the single mutants showed that the double mutant profile was similar to that of *knat7* in many instances, with no obvious additive effect due to double loss-of function. However, there were some differences observed in double mutant relative to *knat7* (Figure 3.11B).

3.5 DISCUSSION

Several transcription factors whose expression in the Arabidopsis inflorescence stem is correlated with the secondary cell wall deposition were identified in an earlier microarray study (Ehlting, et al., 2005). Among these, MYB63 (Zhou, et al., 2009) and KNAT7 (Zhong, et al., 2008; Brown, et al., 2005; Li, 2009) have since been demonstrated to be regulators of secondary wall formation. In addition, secondary cell wall-associated pathway regulators like MYB75 have also been shown to regulate secondary cell wall formation in Arabidopsis stems (Chapter 2). In this work, protein-protein interaction studies identified interaction between some of these known secondary cell wall regulators. Since transcription factors are believed to often exert their regulatory activity through participation in multi-protein complexes, these interactions may represent the partial complexes involved in secondary cell wall formation in Arabidopsis. In particular, I have demonstrated that KNAT7 and MYB75 can interact at the protein level and may therefore form a biologically relevant complex *in planta*.

Little functional information is available about homeodomain-MYB transcription factor interactions, with an exception of one report (Timmermans, et al., 1999) about the interaction of MYB and KNOX in lateral organ primordia in maize. Given that KNAT7 (Zhong, et al., 2008; Brown, et al., 2005; Li, 2009) and MYB75 (Chapter 2) both play a role in secondary wall formation, the *in vitro* and *in vivo* interaction I observed between KNAT7 and MYB75 suggested that both of these proteins could work as a part of a KNOX-MYB complex to regulate some aspects of secondary wall formation in Arabidopsis stem. If MYB75 functions together with KNAT7 as part of a regulatory complex *in vivo*, *MYB75* would be expected to have expression patterns that overlap with those of *KNAT7*. I observed *promMYB75:GUS* expression in the vascular bundle and cortex in the inflorescence stem (Chapter 2) which is similar to the previously reported *promKNAT7:GUS* expression pattern (Li, 2009). This indicates that the MYB75 protein is likely to be present in the same cell types as KNAT7 in certain tissues. However, their coordinated presence in all secondary cell wall-associated cell types remains to be determined.

MYB75 is not only involved in regulating secondary cell wall biosynthesis but also is an important TF in the regulation of carbon redistribution in the phenylpropanoid pathway (Chapter 2). It is noteworthy that the interaction of MYB75 with TT8, a bHLH protein (Zimmermann, et al., 2004), was previously shown to involve a multi-protein complex that positively regulates anthocyanin production (Gonzalez, et al., 2008; Borevitz, et al., 2000). MYB75 also negatively regulates the lignin-specific pathway (Chapter 2) and was shown to interact with KNAT7 to regulate secondary cell wall biosynthesis in this work. This suggests that different complexes may operate at the protein level to regulate two distinct pathways in two different modes (positive and negative). In short, when MYB75 (a weak activator, Chapter 2) interacts with TT8 (an activator; data not shown), this results in a positive regulation of the anthocyanin pathway but when MYB75 interacts with KNAT7 (a repressor, (Li, 2009)), this results in the repression of a set of the secondary cell wall-specific genes. The relationship of these different interactions with contrasting and diverse biological functions provides further evidence of the diversity that protein-protein interaction-based complexes can provide when a limited number of TFs is engaged. In addition, my observations are consistent with a scenario in which multiple complexes might share specific sets of transcription factors, thus providing a rich palette of combinatorial diversity for cross-regulating different metabolic pathways as both activators and repressors of

transcription. Whether MYB75 might serve as an activator or repressor of transcriptional targets within specific putative complexes *in planta* remains to be clarified. The protoplast system employed here to confirm these interaction yielded a result suggesting an overall repression of the activity of a MYB75-KNAT7 complex (Figure 3.5), consistent with repression of the secondary cell wall genes seen in *myb75-1* plants (Chapter 2).

The yeast two-hybrid assay is a powerful tool for investigating the network of interactions that is formed between proteins involved in particular processes (Walhout, et al., 2000). Although the system offers advantages over biochemical methods, such as rapidity and sensitivity, it still has associated problems. False positives are sometimes generated in two-hybrid screens. However, the use of two or more reporter genes (Fashena, et al., 2000) to assay for an interaction, as I used here, has been reported to provide more selectivity and to allow detection of weaker interactions. Interestingly, there were some interactions seen in BiFC experiments that were not observed in yeast two-hybrid assays with the same clones (Figure 3.6 and Figure 3.7). One possible reason for this difference may be the use of a heterologous system like yeast cells for identifying *in vitro* interactions among Arabidopsis proteins. Using an *in vivo* system involving Arabidopsis protoplasts to confirm the interactions is therefore considered to be a better approach, since it might avoid non species-specific unidentified interactions. Thus, I confirmed all of my Y2H interactions in protoplasts using two different experimental approaches. These included BiFC, which have been used extensively in identifying interactions among Arabidopsis proteins (Weinthal and Tzfira, 2009; Schutze, et al., 2009; Walter, et al., 2004), along with a more quantitative method, the protoplast transfection system (Wang, et al., 2007). Data from these two different *in vivo* systems consistently indicated the occurrence of MYB75-KNAT complex formation *in planta*.

Protein-protein interactions are known to be dependent upon different domains, and multi-protein binding models can include the binding of one TF to one domain of a given protein while another TF (or other interactor) binds to another domain. The competition between these binding domains for available partners may provide another level of regulation. For example, the R3 domain of MYB's has been shown earlier to interact with bHLH proteins, while my findings shows that R3 may also be an important domain interacting with KNOX proteins. The negative regulatory functions of R3 proteins is dependent on the ability of these proteins to interact with bHLH proteins due to their competition for binding with the R2R3-MYB partners and not

binding themselves to DNA stably (Dubos, et al., 2010). A similar function for the MYB75-KNAT7 interaction cannot be ruled out. Moreover, it was shown here that the R3 domain interacts specifically with the KNOX2 domain of KNAT7. This observation is an important initial step in determining the structural and sequence specificity of the protein-protein interaction. The precise characterization of amino acid motifs involved in such interactions is important to understand the functional specificities within protein families. It will be interesting in the future to determine which amino acids are specifically involved in the MYB75-KNAT7 interaction. My findings reconfirm the established view for MYBs to not only involve in DNA binding, but also their involvement in protein–protein interactions. Multiple binding capacity of one domain such as R3 to members of different protein families (bHLH, KNOX) supports the idea that recruitment of variety of accessory proteins can be achieved by MYB domain.

The concept of a biological function for MYB75 in regulating secondary wall formation through its interaction with KNAT7 is further supported by the phenotypes of the *myb75-1* single mutant and the *myb75 knat7* double mutant. Both mutants exhibited inflorescence stem phenotypes similar to that of *knat7* (Brown, et al., 2005; Li, 2009) with characteristic enhanced thickness of interfascicular fiber cell walls (Figures 3.8). The lack of an additive phenotype in the double mutant, as seen in light microscopy, transmission electron microscopy and secondary cell wall-associated gene expression, is consistent with the view that KNAT7 and MYB75 may function in the same pathway or in a common complex. I did not observe in the *myb75-1* mutant any indication of an *irx* wall phenotype such as is seen in *knat7* or *myb75 knat7* plants (Figure 3.8). Therefore, it is possible that the putative MYB75-KNAT7 complex is only involved in a subset of *knat7* functions. While MYB75 can interact with KNAT7 *in vivo,* and they share a similar expression pattern and may function as a MYB-KNOX complex regulating secondary wall formation, they may also participate in other pathways and/or possess a very cell-specific complex functionality and localization. Since KNAT7 function seems to be primarily restricted to the developing secondary walls (Zhong, et al., 2008; Li, 2009), in contrast to the broader function of MYB75 (Tohge, et al., 2005a; Teng, et al., 2005; Lea, et al., 2007; Gonzalez, et al., 2008), the mechanism by which MYB75-KNAT7 interactions may pleiotropically impact broader plant developmental processes is unknown and will be the subject of future investigations.

My data supports a model in which MYB75-KNAT7 protein-protein interaction is of particular importance to the regulation of secondary wall formation. This work is unique in

characterizing an interaction between KNOX and MYB family protein and identifying its role in regulating secondary cell wall formation in Arabidopsis inflorescence stems. It can also be postulated that there are likely to be more partners involved in this interaction and that *in planta* identification of the relevant protein complexes will be necessary in order to more fully understand the complete biological regulatory network.

Chapter 4. MYB75-KNAT7 protein interaction in the context of regulating secondary cell wall formation in the Arabidopsis seed coat

4.1 SYNOPSIS

Arabidopsis seeds possess extensive secondary walls in their mature seed coat, where they are deposited in the radial cell walls and columella of the sub-epidermal layer. In addition to strengthening the seed coat, these structures provide protection for the cells involved in mucilage production and storage. MYB75 and KNAT7 were shown earlier to interact physically and genetically, and to be involved in the regulation of some aspects of secondary cell wall formation in the Arabidopsis stem (Chapter 3). Here, I show that at least some parts of the transcriptional regulatory network involved in the formation of secondary cell walls in the Arabidopsis inflorescence stem are functionally conserved in the seed coat and that the seed coat development network involves the putative MYB75-KNAT7 protein complex. Novel genetic and protein-protein interactions of MYB75 and KNAT7 with other transcription factors known to be involved in seed coat regulation were also identified, leading to the proposal that a MYB75-associated protein complex is likely to be involved in regulating secondary cell wall biosynthesis in the seed coat.

4.2 INTRODUCTION

Secondary cell walls in plants provide mechanical strength, support and protection. They are found in many different organs and tissues, including inflorescence stems, leaves, roots and seed coat. The seed coat is a shielding structure that protects the embryo and also contributes to seed dormancy, dispersal and germination. It is derived from the ovule integuments following fertilization. Overall seed coat development can be divided into three main stages (Haughn and Chaudhury, 2005) (Figure 4.1). The first stage (3-5 days post fertilization/anthesis (DPA)) includes five different layers of the ovule integument undergoing a period of growth (1-5, Figure 4.1A). In the second stage, these layers differentiate toward specific fates, a process that occurs

within 7-11 DPA. Differential changes that occur during this stage include synthesis of condensed tannins by the endodermis layer ((5), Figure 4.1B), crushing of two inner integument layers ((3) and (4), Figure 4.1B), production of a thickened secondary cell wall on the inner tangential side of the cells by the subepidermal layer ((2), Figure 4.1B) and synthesis and secretion of a large quantity of mucilage (a pectinaceous carbohydrate) by the epidermal layer into the apoplast, specifically at the junction of the outer tangential and radial cell walls ((1), Figure 4.1B). Further, contraction of the vacuole takes place because of mucilage deposition that results in a column-like structure in the centre of the cell, called the columella. This structure is later surrounded by the deposition of a secondary cell wall that completely fills the apoplastic space (Figure 4.1C): therefore, the main areas of secondary cell wall deposition during the differentiation process of the seed coat development are radial cell walls and the walls of the cytoplasmic columns (Haughn and Chaudhury, 2005; Beeckman, et al., 2000; Western, et al., 2000). In the mature stage of differentiation, the cells of all seed coat layers die. Mucilage and columella preserve the structure of the epidermal cells as the remaining layers are crushed together (Figure 4.1C).

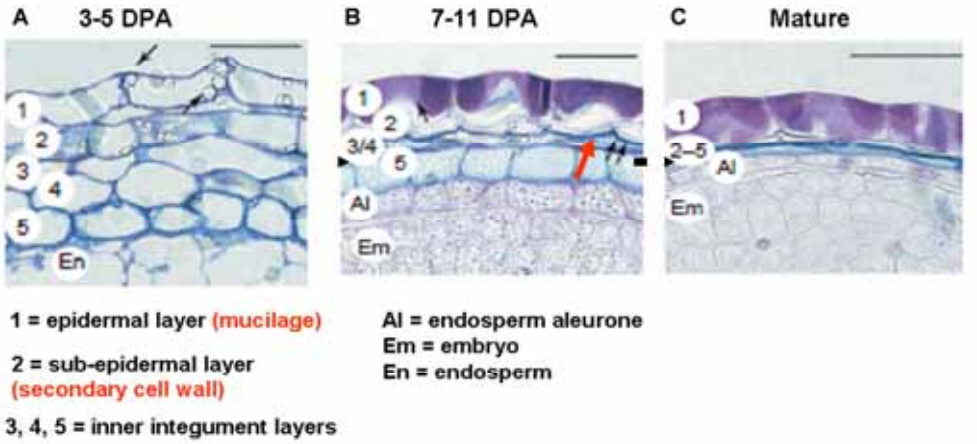


Figure 4.1 Development of the ovule integuments into a seed coat. Three main stages during the development are shown (A) 3-5 DPA (Days Post Anthesis/fertilization) (B) 7-11 DPA and (C) mature stage. The cell layers of the ovule integument undergo a period of growth (3-5 DPA; 1–5). Differentiation of the cell layers and mucilage and secondary cell wall formation and deposition takes place (7-11 DPA). Cells of all layers have been crushed together and are dead. A thick secondary cell wall of the columella is seen (Mature). Red arrow indicates area of secondary cell wall deposition. Al, endosperm aleurone; Em, embryo; En, endosperm. (Adapted from Haughn and Chaudhury, 2005).

The differentiation of the seed coat involves regulation by transcription factors (TF). Many transcription factors from different families have been demonstrated to influence mucilage/secondary cell wall production in Arabidopsis seeds. The distinction between regulation of the mucilage production pathway(s) and secondary cell wall-specific pathway(s) is not very clear, since many TFs have been proposed to be involved in regulating both of these networks (Li, et al., 2009b; Gonzalez, et al., 2009). The most prominent transcription factors involved include APETALA2 (AP2) a homeodomain transcription factor needed for differentiation of the outer layers of the seed coat as well as transparent testa glabra 1 protein TTG1 (a WD repeat protein) and GLABRA2 (GL2, a homeodomain TF), that are required for general differentiation (Haughn and Chaudhury, 2005; Western, et al., 2004; Western, et al., 2001). A complex containing TTG1, two bHLH family members (EGL3-ENHANCER OF GLABARA 3, TT8-TRANSPARENT TESTA 8) and two MYB proteins (MYB5, MYB23) is believed to regulate the biosynthesis of mucilage. (Li, et al., 2009b; Matsui, et al., 2004; Gonzalez, et al., 2009; Baudry, et al., 2004). In the seed coat, TTG2 (TRANSPARENT TESTA GLABRA 2; a WRKY TF), TT2 (TRANSPARENT TESTA 2) and MYB61 (MYB TFs), and GL2 and KNAT7 (KNOTTED ARAPIDOPSIS THALIANA 7; homeodomain TFs) have all been shown to regulate some aspects of mucilage and/or epidermal development (Li, et al., 2009b; Li, 2009; Haughn and Chaudhury, 2005; Ramsay and Glover, 2005; Western, 2006). Genetic interactions have been previously reported to involve many of these secondary cell wall formation/mucilage biosynthesis genes and different regulatory network models have been proposed (Haughn and Chaudhury, 2005; Gonzalez, et al., 2009).

In addition to regulating seed coat secondary cell wall/mucilage biosynthesis, some of these same transcription factors have also been implicated in other developmental processes and pathways. For example, anthocyanin biosynthesis is regulated by TT8, GL3, EGL3 and TTG1, in the seed coat, proanthocyanidin (condensed tannin) biosynthesis regulation is done by TT2, TT8, and TTG1 and KNAT7 and MYB61 have been associated with cell wall regulation and sugar sensing, respectively (Dubos, et al., 2010; Li, 2009). The divergence in biological functions of these genes could be the result of differential genetic interactions (e.g. spatial and/or temporal diversity in expression patterns) and/or of the formation of different protein complexes that modify the cellular activities of the gene products.

Transcription factors are thought to generally work in complexes and therefore, protein-protein interactions are important for their functions. WD40 repeat proteins form complexes with basic helix-loop-helix (bHLH) transcription factors and with many MYB transcription factors to regulate different developmental processes (Schellmann and Hulskamp, 2005; Serna and Martin, 2006; Serna and Martin, 2006; Serna, 2005; Guimil and Dunand, 2006; Martin and Glover, 2007) and metabolic pathways in plants (Haughn and Chaudhury, 2005; Ramsay and Glover, 2005; Western, 2006; Broun, 2005; Koes, et al., 2005). Similarly, homeodomain (HD) transcription factors (e.g. KNOX family members) also show protein-protein interactions with BELL (Bellaoui, et al., 2001; Viola and Gonzalez, 2006), OFP proteins (Li, 2009; Hackbusch, et al., 2005) and MYB family proteins (Chapter 3) and HD proteins are involved in regulation of many different metabolic pathways, including secondary cell wall formation in Arabidopsis. Transcription factor protein–protein interactions have sometimes been shown to be conserved in different tissue types (Gonzalez, et al., 2008; Baudry, et al., 2004; Nesi, et al., 2001; Nesi, et al., 2000), but conservation of such interactions across tissue types for transcription factors involved in regulation of secondary cell wall formation has not previously been examined in the literature.

MYB75 regulates many metabolic pathways, including anthocyanin biosynthesis (Gonzalez, et al., 2008; Borevitz, et al., 2000), and has been shown to be a negative regulator of secondary cell wall formation in the Arabidopsis inflorescence stem (Chapter 2). Physical interactions between MYB75 and the bHLH TFs, TT8 and bHLH012 (Zimmermann, et al., 2004) have been reported earlier. KNAT7, a KNOX family protein has been shown to regulate xylogenesis (Brown, et al., 2005) and secondary cell wall biosynthesis in Arabidopsis (Li, 2009). It also shows protein-protein interactions with OFP family proteins (Li, 2009; Hackbusch, et al., 2005) and BEL1-like homeodomain (BLH) family proteins (Hackbusch, et al., 2005). Protein-protein interaction between KNAT7 and MYB75, and the role of these interacting proteins in regulating secondary cell wall formation in the Arabidopsis inflorescence stem was examined in Chapter 3.

In this chapter, I show the possible involvement of MYB75-KNAT7 protein-protein interaction in regulating secondary cell wall formation in seed coat radial walls and columella similar to the role of this interaction that has been shown earlier in the regulation of secondary cell wall thickening in the inflorescence stem (Chapter 3) of Arabidopsis. Further, the question whether this regulation is mediated by interaction with a distinct set of transcription factors in

seed coat was examined. Based on my findings, I have been able to position KNAT7 and MYB75 within the known genetic interaction matrix involved in regulating the mucilage/seed coat wall biosynthesis pathway(s). I also propose that a distinct complex involving these two transcription factors is likely to contribute to regulation of secondary cell wall formation in the Arabidopsis seed coat.

4.3 MATERIALS AND METHODS

4.3.1 Plant material

The loss-of-function MYB75 mutant (*myb75-1*) (Chapter 1) used in this work is in the Arabidopsis ecotype Nossen background. These seeds were compared to wild-type (WT) Nossen seeds. Seeds of the KNAT7 (*knat7*) loss-of-function mutant were a gift from Eryang Li, UBC (Li, 2009). This mutant is in the Columbia background and so was compared to wild type Columbia seeds in this work. The double-loss-of-function mutant (*myb75 knat7*) (chapter 3) was compared to both wild types (Nossen and Columbia). Homozygous seeds of a TT8 loss-of-function mutant allele (*tt8*) obtained from the Arabidopsis Biological Resource Center (ABRC) were used as a control in this work. The other seed coat mucilage mutant seeds (*ap2, gl2, ttg1* and *ttg2*) were a kind gift from Dr. George Haughn (Botany department, UBC). For protoplast isolation, WT Columbia seeds were surface sterilized using 20% commercial bleach, cold-treated at 4°C in the dark for 2 days and plated on agar ½ MS medium (2.16 g/l MS salts, 1% sucrose, 1% bacto-agar pH 6.0 with 1 M KOH (Murashige and Skoog, 1962). Ten-days-old seedlings were grown in a moistened 1:3 mixture of Sunshine Mix #1 (Sun Gro Horticulture Canada Ltd) and with a 16/8 h (light/dark) photoperiod at 23°C. Leaves from plants that were approximately 3–4 weeks old were used for protoplast isolation. For seed coat isolation at specific development stages of the seed, WT (Columbia and Nossen) and mutant seeds (*ap2, gl2, ttg1, ttg2, myb7* and *knat7*) were grown in soil as described above for protoplast isolation. Plants were monitored for their silique development and stage-specific seed coat tissue was collected as described below.

4.3.2 Stage-specific seed coat separation, RNA isolation and qRT-PCR

For developmental stage-specific seed coat isolation, a stage was defined as the time when the flower was beginning to open (days post anthesis; DPA). Flowers at anthesis were labeled using

appropriate paint, and using a different color for each stage. Siliques were collected, pooled together and then used for RNA isolation. Total RNA was extracted from 3-, 7-, and 11-DPA WT or mutant seed coats using the RNAqueous kit (Ambion). To eliminate residual genomic DNA, the RNA was treated with RNAse-free DNAseI according to the manufacturer's instructions (Qiagen). The concentration of RNA was measured using the absorbance at 260 nm and the quality of the sample was assessed using the A260/A280 ratio. Total RNA (100 ng) was reverse transcribed using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. 1.5 µl cDNA was used in each reaction in a 20 µl reaction volume. PCR amplification was performed with *Knat7*-, *MYB75*-specific primers, using *Actin8* as a normalization control. *Actin8* was chosen as the reference gene for this study as its expression has been shown to be relatively invariant (An, et al., 1996). However, in my experiments, stable expression of *Actin8* was reconfirmed in seed coat-specific samples (data not shown). The cDNA was amplified using the PerfeCta™ qPCR FastMix (Quanta Biosciences) on the DNA Engine Opticon® 2 (Bio-Rad). Relative values are arbitrary units and were calculated as described previously (Gutierrez, et al., 2008).

4.3.3 Scanning electron microscopy and image analysis

Standard EM stubs were used and Arabidopsis seeds (WT and mutants) were attached and sputter coated with gold-palladium alloy. Hummer VI sputtering system (Anatech) was used for this purpose. Hitachi (S-800) scanning electron microscope was used to visualize the specimens and images were taken using a digital imaging system.

4.3.4 Bright field microscopy and resin embedding

Mature seeds were fixed in 3% glutaraldehyde as described by Stork et al., (2010). All samples were slowly infiltrated with Spurr's epoxy resin after transferring to propylene oxide solution. Dehydration, embedding and sectioning were performed as described by Western et al., (2000). Axioskop 2 microscope (Carl Zeiss) was used to photograph the sections. Height and width of the columella and radial wall were determined as described by Stork et. al., 2010. A total of atleast

10 cell walls were measured for each genotype. Student's t-test was used to determine the significance.

4.3.5 Histochemical analyses

0.2% (w/v) Ruthenium red (Sigma, St. Louis, MO) was used to test for abnormal mucilage production from the seeds, according to Beeckman et al., (2000). Dye was dissolved in water for 30 min at 25°C. The seeds were photographed with an Axioskop 2 microscope (Carl Zeiss).

4.3.6 Yeast two-hybrid assays

The ProQuest yeast two-hybrid system (Invitrogen) was used with full length transcription factors in pDEST32 (bait vector) or pDEST-22 (prey vector) introduced into the yeast strain MaV203 in different combinations. Positive clones were isolated on the basis of three selectable markers: *HIS3, URA3* and *LacZ*. Positive interactions were indicated by activation of *HIS3* or *URA3*, according to the manufacturer's instructions

4.3.7 Protoplast isolation and bi-molecular fluorescence complementation (BiFC) using YFP

The procedures for Arabidopsis protoplast isolation has been described previously (Wang, et al., 2007). Gateway entry vectors with full length MYB75, KNAT7, MYB5 and TT8 were used. For N-terminal YFP-tagged constructs, appropriate entry clone was transferred into BiFC expression vector pSAT4-DEST-nEYFP$_{1–174}$-C1 (pE3136) or pCL112 (pBATL) to produce nYFP–vectors. The same procedure was used for C-terminal YFP-tagged constructs using pSAT5-DEST-cEYFP$_{175–end}$-C1(B) (pE3130) or pCL113 (pBATL) to produce cYFP–vectors. The resulting plasmids were co- transfected into freshly prepared Arabidopsis leaf mesophyll protoplasts, and incubated for 20–22 hours (Wang, et al., 2007). YFP fluorescence was visualized using a Leica DM-6000B fluorescence microscope and photographed with a Leica digital image system (Leica Microsystems).

4.3.8 Primers

Primer sequences for real time PCR (qRT-PCR) were: MYB75 left primer, 5′-TTCTTCGCCTTCATAGGCTT-3′, right primer, 5′-AGGAATGGGCGTAATGTCTC -3′; KNAT7 left primer, 5′-AAATTGGTGGAGGAGACAGG-3′, right primer, 5′-TCCTCTTGCGTTGGTTAATG-3′; ACT8 left primer, 5′-TCTAAGGAGGAGCAGGTTTGA-3′, right primer, 5′- TTATCCGAGTTTGAAGAGGCTAC -3′.

4.4 RESULTS

4.4.1 MYB75 and KNAT7 are expressed in seed coat stages involving active secondary cell wall formation and are part of the transcriptional network regulating Arabidopsis mucilage/ seed coat development

To identify whether *MYB75* and *KNAT* are expressed in seed coat developmental stages where secondary cell wall deposition takes place (7-11 DPA, Figure 4.1) and to establish whether these genes might form part of the known genetic network regulating secondary cell wall/mucilage pathway, their expression was assessed using quantitative real-time PCR (q-RT-PCR)(Figure 4.2). Transcript quantifications were done using cDNA from stage-specific seed coats (3, 7 and 11 DPA) isolated from WT plants and plants carrying mutations in known seed coat regulatory genes. *MYB75* and *KNAT* transcript levels were found to be high in 7 and 11 DPA stages as compared to 3 DPA (Figure 4.2A and 4.2B). This indicated that these two genes could potentially be involved in regulating secondary cell wall deposition in the seed coat. Further, *MYB75* expression was strongly suppressed in the *ttg2* background (Figure 4.2A), indicating that *MYB75* is associated with a genetic network known to be regulating seed coat secondary cell wall/mucilage deposition and *TTG2*, one member of that network, is upstream of *MYB75*. However, no changes were observed in *MYB75* expression in the other mutant backgrounds examined (*ap2, gl2 and ttg1*). Based on this observation, we can predict that *MYB75* serves as a downstream target of *TTG2* but that this relationship may be independent from the pathways previously known to be regulated by *TTG2* (Figure 4.6A). It is noteworthy that no change in *KNAT*7 expression was observed in *myb75* seed coats relative to that seen in WT plants (Figure 4.2B). On the other hand, *KNAT7* expression was higher in the *ttg2* background compared to WT,

indicating negative regulation (repression) of *KNAT7* expression by *TTG2* (Figure 4.2B). Expression of KNAT7 was also downregulated in the *ap2* and *gl2* backgrounds, suggesting that *KNAT7* is situated downstream of *AP2*, *GL2* and *TTG2* in the known genetic interaction network and may be a part of the mucilage biosynthesis pathway (Li, 2009).

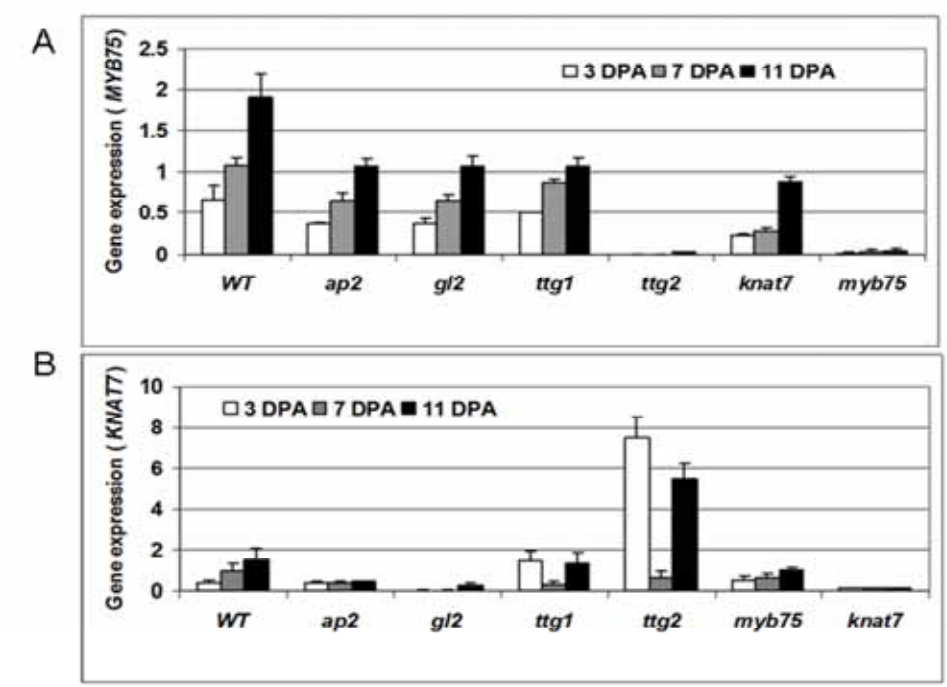


Figure 4.2 Expression of *MYB75* and *KNAT7* in mutants of putative regulatory transcription factors during seed coat development. Total RNA was isolated from 3-DPA,7-DPA and 11-DPA stages of seed coat development of wild-type, *ap2, gl2, ttg1, ttg2*, *myb75* and *knat7* siliques. cDNA was used for quantitative real time PCR (q-RT-PCR) using gene-specific primers for *MYB75* (A) and *KNAT7* (B).

The expression of *KNAT7* in the *ttg1* and *ttg2* backgrounds also shows a bimodal distribution of expression during stage-specific development (Figure 4.2 B). From a gene expression standpoint, a bimodal distribution of *KNAT7* expression suggests the potential existence of two sets of mRNA populations in 3 DPA and 11 DPA stages of seed coat development, and that these are differentially regulated by TTG1 and TTG2. This pattern indicates that these *KNAT7*-associated mRNA sets may be playing two different roles in each stage, with one (3 DPA) involved in growth and development of the epidermal layers and other (11 DPA) more specific for its role in late differentiation stage (mucilage and secondary wall formation).

4.4.2 MYB75 and KNAT7 interact to regulate secondary cell wall in Arabidopsis seed

Examination by scanning electron microscopy (SEM) was performed on WT (Columbia and Nossen), *myb75*, *knat7,* and *myb75 knat7* seeds (Fig. 4.3A) to gain insight into the seed coat surface and cell shape phenotype in these mutants. Surface structures of the seed coat were irregular in the mature *myb75* seed. In this mutant, the angle between the radial wall junctions was distorted and, in general, only four walls were visible as compared to symmetrical six-walled surface structures in WT seed coats. Further, the radial cell walls were thicker in *myb75* seed coats as compared to WT (Nossen) seeds. In *knat7* seeds, the radial wall also appeared to be thicker relative to the WT (Columbia) (Figure 4.3A). However, no change in the hexagonal cell wall shape was evident in the *knat7* seeds. In *tt8* mature seeds, which were used as a positive control in this study, distorted and thinner epidermal cells were observed (Figure 4.3A.). Genetic interaction between *MYB75* and *KNAT7* was suggested by the enhanced but intermediate phenotype seen in *myb75 knat7* seed coats relative to the single mutants or WT; the *myb75 knat7* double mutant had thicker radial walls, a higher columella, distorted radial wall angles and an overall cell shape marked by four visible walls, features that are similar to those seen in *myb75* seed coats (Figure 4.3A). Based on these phenotypic data, it is likely that *MYB75* and *KNAT7* genes operate in similar pathway(s) and that their potential interaction could play a role in regulating secondary wall thickness in Arabidopsis seed coat epidermal cells.

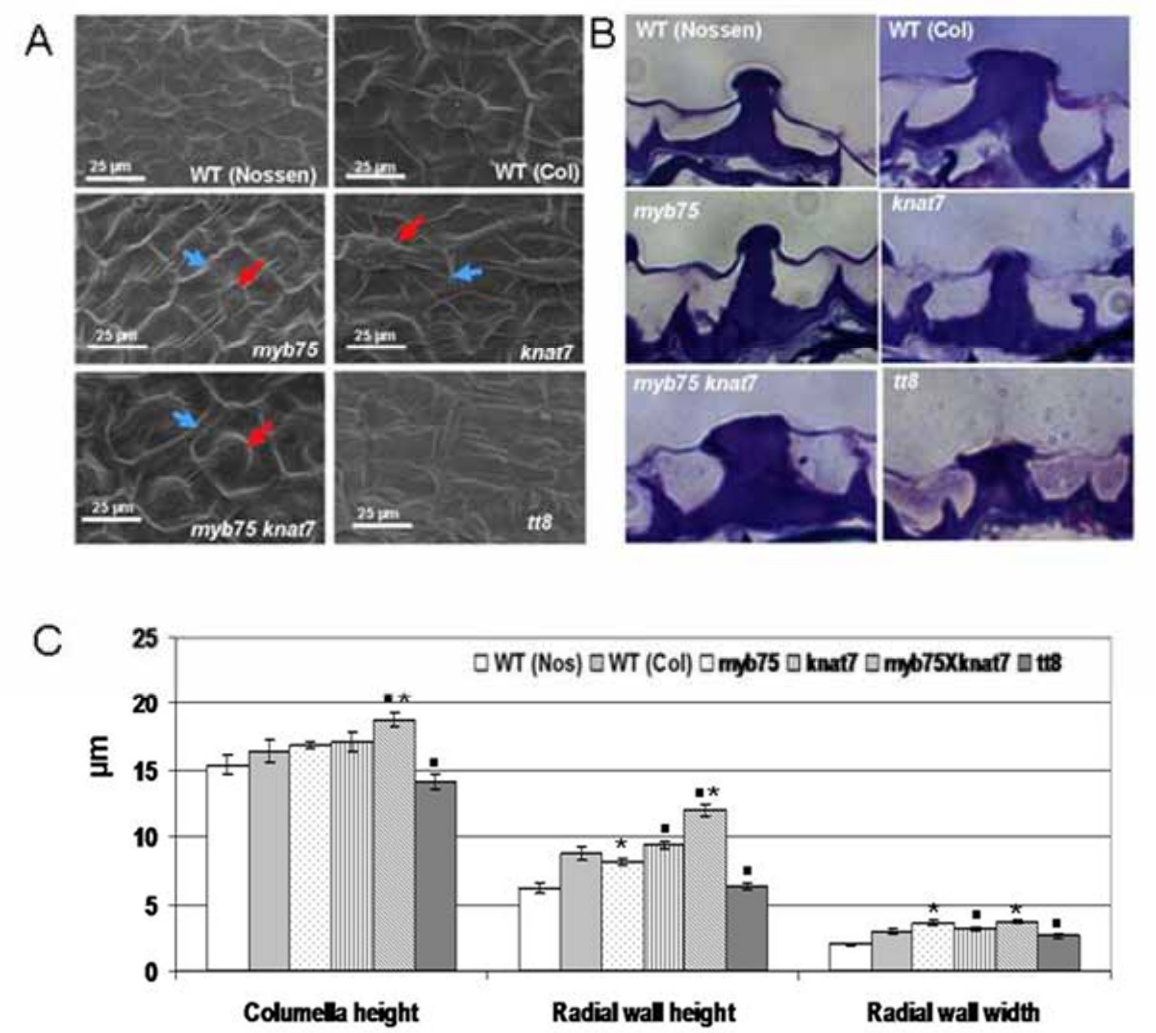


Figure 4.3 Mutations in MYB75 cause change in cell shape and thickening of radial walls in seed coat epidermal cells (A) Scanning electron microscopy of dry seeds of wild type (Columbia and Nossen), *myb75, knat7, myb75 knat7* and *tt8* mutants (as indicated). The changes in hexagonal cell walls and columella in the centre of WT cells in mutants is noticeable. Radial wall and columella are the regions of secondary cell wall deposition. Blue arrow indicates change in radial wall and red arrow indicates columella (B) Epidermal cell morphology of wild-type (Columbia and Nossen), *myb75, knat7, myb75 knat7* and *tt8* toluidine blue-stained sections of mature seeds (as indicated). (C) Quantification of the radial wall height, width and columella length of wild-type and mutants seed coat cells (µm). Error bars are SE from the mean. Square (▪) indicates significant difference from WT-Col. Asterisk * indicates significant difference from WT-Nos. *myb75* is in Nossen background and *knat7* and *tt8* are in Columbia background.

To quantify the SEM-observed phenotype, histological analysis was performed on Toluidine Blue-stained sections of mature seeds of WT (Columbia and Nossen) and mutants (*myb75, knat7*, *myb75 knat7* and *tt8*) (Figure 4.3B). In the experimental conditions used in this work, mutant and

wild-type seeds were fixed under aqueous conditions (3% glutaraldehyde). This specific treatment caused the bursting of the mucilage pocket. This resulted in exposure of the epidermal secondary cell wall. Variation in the height and width of the *myb75* radial wall was clearly evident. However, cell wall appeared larger than in the wild type (Figure 4.3B). To further quantify these changes, the radial wall height and width were measured. The results (Figure 4.3C) indicated significant differences in the height and width of the *myb75* and *myb75 knat7* radial walls when compared to wild-type cells. An increase in radial wall thickness was also observed in *knat7* mutant seeds (Figure 4.3C).

Based on the SEM, histological studies and the phenotype observed in *myb75 knat7* seed coats, which show more similarity to *myb75,* it is possible that both *MYB75* and *KNAT7* belong to the same pathway/complex to cooperatively regulate secondary cell wall formation in the Arabidopsis seed coat.

4.4.3 *MYB75* seeds do not have a seed mucilage phenotype

In addition to the extensive secondary cell wall deposition in the Arabidopsis seed coat, secretion of abundant pectin-rich mucilage takes place during the differentiation stage. Therefore, both the secondary cell wall deposition and mucilage formation are important and simultaneous processes during seed coat development. To determine the role of MYB75 and KNAT7 in the mucilage biosynthesis pathway, I examined *knat7, myb75* and *myb75 knat7* seeds for possible mucilage defects, which can be easily observed following imbibition (Figure 4.4). When wild-type Arabidopsis seeds contact water, they release mucilage from the seed coat epidermis, and this expands to form a gelatinous coating over the seeds (Western, et al., 2000). This mucilage coating can be visualized by staining with Ruthenium Red, which stains negatively charged polymers such as pectin and DNA (Koornneef, 1981)(Figure 4.4A and 4.4B). When stained with Ruthenium Red after shaking (the treatment disturbs the pectin network of the mucilage) in water, there was barely a visible layer of stained mucilage observed in *knat7* seeds (Figure 4.4D). However, when *knat7* seeds were placed directly in water and stained without agitation, a dense layer of mucilage was apparent. Since Ruthenium Red staining is dependent on pectin composition (Sterling, 1970), this behavior indicates a difference in mucilage composition between WT and *knat7* mutant seeds, supporting the idea that KNAT7 might play a role in

regulating mucilage biosynthesis. In *myb75* seeds, a dense mucilage layer was observed around seeds imbibed either with or without shaking (Figure 4.4C). Therefore, it appears that MYB75 does not play an essential role in regulating either mucilage biosynthesis or composition and that it may be specialized as a regulator of secondary cell wall formation in the seed coat.

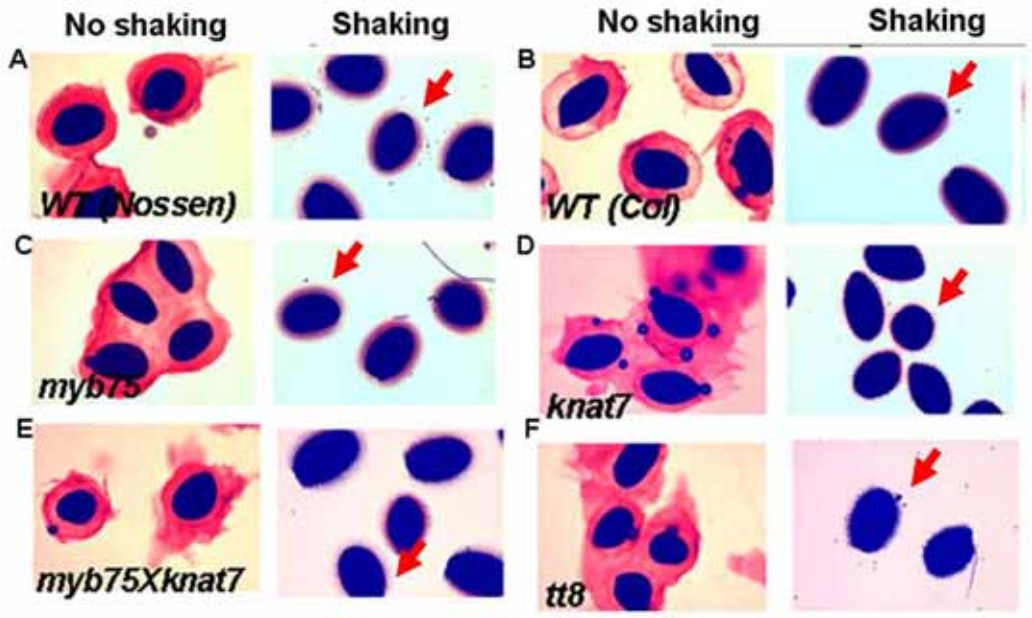


Figure 4.4 Mucilage release by *myb75* and *knat7* seeds compared to wild-type seeds imbibed in Ruthenium Red solution, with and without shaking. (A) Nossen wild-type (B) Columbia wild-type seeds. (C) *myb75* seeds. (D) *knat7* seeds. (E) *myb75 knat7* seeds. (F) *tt8* seeds. *myb75* and *myb75 knat7* seeds (C and E) display normal mucilage release (as seen in WT) when treated directly with Ruthenium Red, with or without agitation, while *knat 7* and *tt8* seeds (D, F) shaken in dye lose the soluble layer of mucilage. Red arrows indicate the mucilage layer.

4.4.4 MYB75 and MYB5 show protein-protein interaction with KNAT7

To identify potential protein-protein interactions among MYB75, KNAT7 and some of the known seed coat differentiation regulating TFs, I assayed them against each other in directed yeast two-hybrid (Y2H) assays (Figure 4.5A). All the tested candidates were fused to the GAL4 DNA binding domain (BD) and GAL4 activation domain (AD) and assayed for their ability to bind to themselves, or to each other, using a Proquest Y2H kit (Invitrogen). Wild-type yeast was transformed with yeast two-hybrid BD- or AD- construct. Different combinations were generated. Interaction was assessed using yeast growth on dropout media as a reporter. Yeast

growth above the background on SC-Leu-His in the presence of at least 25 μM 3AT and growth on SC-Leu-Ura plates was considered a positive result. MYB constructs were not used as binding domain fusions in the yeast two-hybrid system because of their inherent transcription activating function. Positive Y2H interactions were observed between MYB5 and KNAT7 (Figure 4.5A). MYB5 was shown previously to regulate seed coat mucilage biosynthesis (Li, et al., 2009b). MYB75-TT8, MYB75-bHLH012 (Zimmermann, et al., 2004) and MYB75-KNAT7 (Chapter3) have been previously shown to interact, and since these interactions were corroborated by this assay, they could then serve as positive controls.

The bi-molecular fluorescence complementation assay (BiFC) using a split yellow fluorescent protein (YFP) was used to demonstrate that the MYB5-KNAT7 interaction and other interactions demonstrated in the Y2H system could also be observed *in vivo* in Arabidopsis protoplasts (Figure 4.5 B). MYB5 was fused to N-terminal split YFP and KNAT7 to the C-terminal fragment of the fluorescent protein, neither of which is capable of generating fluorescence alone. Similarly, KNAT7 was fused to N-terminal split YFP and TT8 to C-terminal YFP. A known transcription factor interaction (MYB75 and KNAT7) was used as a positive control in this BiFC experiment (Chapter 3; Figure 4.4). Using the Arabidopsis leaf mesophyll protoplast transient expression system (Tiwari, et al., 2006), I transformed different combinations of fusion constructs. Reconstitution of complete YFP as seen by its fluorescence could be detected in the identified YFP-C-KNAT7 with YFP-N-MYB5 interaction and previously identified interactions *in vitro*: TT8-MYB75, TT8-MYB5 and MYB75-KNAT7 (Figure 4.5B). Only few protoplasts showed positive interaction. This could be the result of lost viability of protoplast before transformation. Constructs with YFP-N-MYB75 with YFP-C-EV were used as a negative control. Therefore, both MYB75 and KNAT7 show protein-protein interactions with other known regulators of seed coat development, suggesting that these interactions may result in formation *in vivo* of different multi-protein complexes that could participate in the regulation of either mucilage or secondary cell wall formation in the developing Arabidopsis seed coat. It can be postulated that the MYB75-KNAT7 pair would operate as one part of such a multi-member complex (Figure 4.6 B).

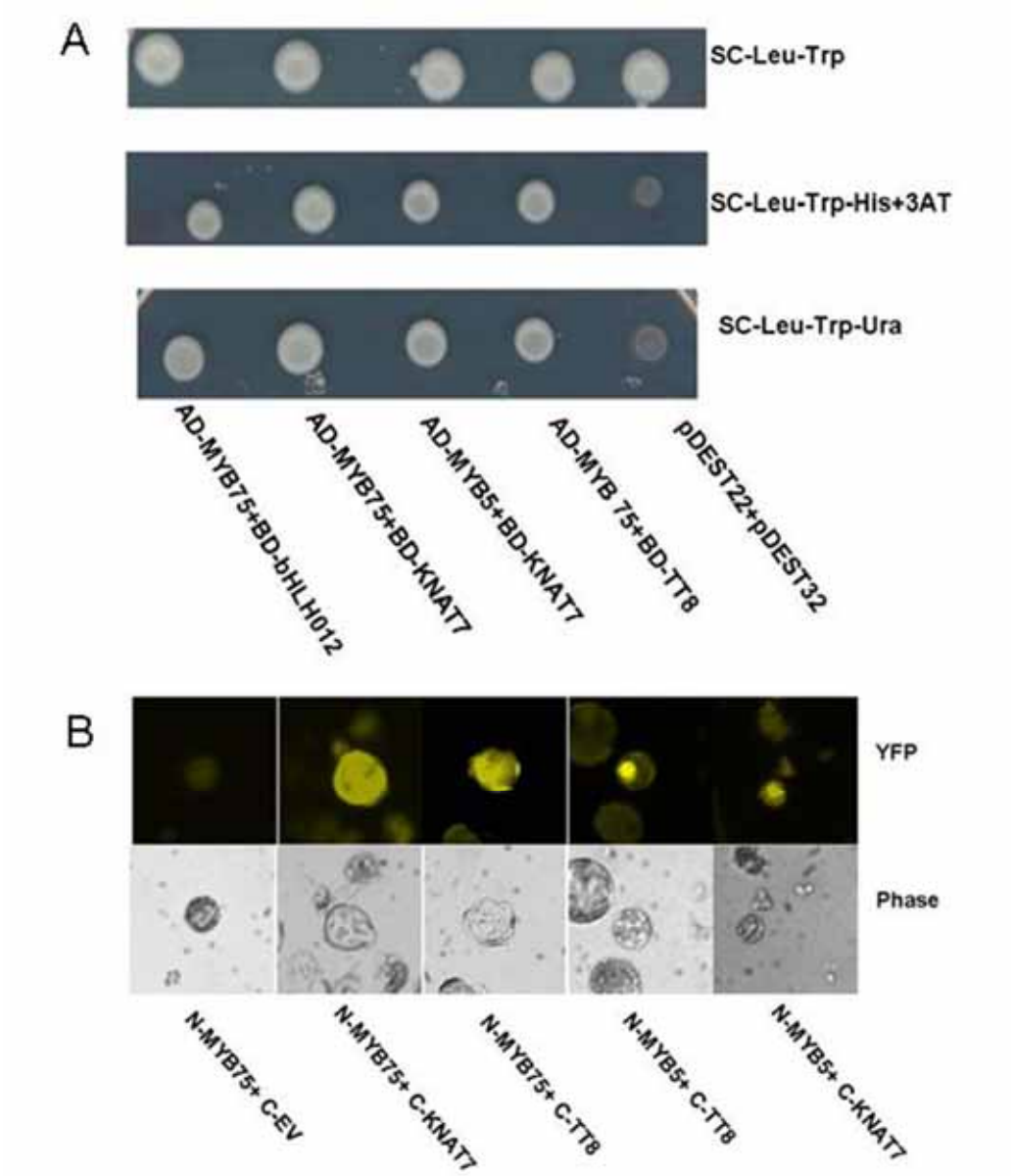


Figure 4.5 In vitro protein–protein interactions among potential secondary cell wall-associated and seed coat regulating as determined by yeast two-hybrid assay (A) and confirmation of interactions *in vivo* by BiFC assay in Arabidopsis protoplast and split YFP (B). Known interactions as indicated in text (Zimmermann et. al, 2004) were used as a positive control and co-transformed empty vectors were used as a negative control.

Based on SEM, histological studies, mucilage staining and protein-protein interaction analyses, the *myb75* and *knat7* mutations appear to affect different but somewhat overlapping aspects of seed coat development, with MYB75 regulating specifically secondary cell wall deposition and KNAT7 having a role in mucilage and secondary wall biosynthesis. However, an interaction

between MYB75 and KNAT7 is conserved in the seed coat and both transcription factors contribute to the regulation of secondary cell wall formation in developing Arabidopsis seeds.

4.5 DISCUSSION

The molecular underpinnings of secondary cell wall deposition as part of seed coat reinforcement remain poorly characterized, including the regulatory role of transcription factors. In this study, I demonstrate a unique and conserved contribution of MYB75 and KNAT7, two interacting transcription factor proteins, to secondary cell wall biogenesis in Arabidopsis seed coat. The pattern of *MYB75* and *KNAT7* expression across specific seed coat development stages in WT Arabidopsis suggest that both of these transcription factors could have a role in this process. High expression is seen in the 11 DPA developmental stages, the stage in which secondary cell wall deposition takes place. This correlation is analogous to the high expression seen for *MYB75* and *KNAT7* in secondary cell wall-associated lower regions of inflorescence stems in Arabidopsis as compared to the upper region of the stem (Chapter 2, Li, 2009). MYB75 and KNAT7 transcription factors were found earlier to play a regulatory role in secondary cell wall formation in the Arabidopsis stem (Chapter 2 and Chapter 3), and it appears that this co-expression of *MYB75* and *KNAT7* is conserved in Arabidopsis in different tissues that have a biological commitment to secondary cell wall deposition. I was also able to show in this work that both *MYB75* and *KNAT7* operate downstream of *TTG2*, a known TF within seed coat development. *TTG2* is situated at an important node in the transcription factor network involved in mucilage biosynthesis and secondary cell wall deposition in seed coat (Li, et al., 2009b; Haughn and Chaudhury, 2005; Gonzalez, et al., 2009; Western, et al., 2004)(Figure 4.6A). *KNAT7* seems to be negatively regulated by *TTG2* (Figure 4.2B) and KNAT7 itself has been shown to be repressor (Li, 2009). Repression of KNAT7 by TTG2 could be an important step in the regulation of secondary cell wall/mucilage formation in the seed coat. In short, based on the data from earlier work and the genetic interaction data gathered in this work, I can propose the position of *MYB75* and *KNAT7* in already known genetic interaction network.

The Arabidopsis seed coat development is regulated by a complex transcriptional network involving number of transcription factors in various combinations to regulate seed coat epidermal layer differentiation and formation of secondary cell wall and mucilage (Li, et al., 2009b; Haughn

and Chaudhury, 2005; Gonzalez, et al., 2009). However, the downstream members in this network that specifically regulate the mucilage or secondary cell wall formation are not known. Both *MYB75* and *KNAT7* may lie downstream of TTG2 to provide functional specificity in the pathway. The double loss-of-mutant (*myb75 knat7*) showed an intermediate seed coat phenotype compared to both single mutants (*knat7* and *myb75*) but with more similarity to *myb75*. This opens up two possibilities: it may indicate that these two TFs belong in the same pathway regulating secondary cell wall in Arabidopsis seeds or belong to separate branches of same regulatory pathway. It seems that in the case of Arabidopsis seed coat, *MYB75* has a dominant epistatic relationship with *KNAT7* for secondary cell wall deposition. This is in contrast to the observation in Arabidopsis inflorescence stem where *KNAT7* showed a dominant role (Chapter 3). This may indicate the spatial-specific effect of the transcription factors but with a conserved interaction. However, this work does not provide enough evidence to rule out the possibility that these TFs may also belong to separate branches of a common regulatory pathway in Arabidopsis seed coat.

Intermediate phenotypes by two genes belonging to distinct sub branches of the main pathway have been shown in many instances in different tissues (Chory, et al., 1991; Diet, et al., 2004; Gomez-Mena, et al., 2001). Therefore, it is possible that either two distinct pathways and/or two separate branches of the same pathway may be involved in the secondary cell wall formation in the seed coat development in Arabidopsis. Further experiments are needed to clarify this ambiguity. The data in this work do suggest that *MYB75* and *KNAT7* appear to regulate a different subset of seed coat development in addition to regulating secondary cell wall formation together as a complex. MYB75 appears to regulate specifically secondary cell wall but KNAT7 having role in both secondary wall formation and mucilage biosynthesis. This postulation is based on the observations in this work on *myb75* and *knat7* mutants. MYB75 loss-of-function mutant did not show any difference in the mucilage when stained with Ruthenium Red. This may be an indication that MYB75, although a part of overall transcriptional network regulating seed coat development (Figure 4.6A), is very specific for secondary cell wall formation pathway in seed coat development. In contrast, KNAT7 also has a role in mucilage biosynthesis pathway as seen by changes in mucilage-specific staining (Figure 4.4; Li, 2009) on mechanical agitation. This brings up the question how this distinction between two pathways (secondary cell wall and mucilage biosynthesis) is generated with the participation of a common transcription factor like

KNAT7? The answer to this question may lie in the genetic interaction of *MYB75* and *KNAT7* with *TTG2* which may indicate distinction provided by *TTG2* in these two branches of the seed coat development. TTG2 has been shown to have role in multiple pathways in seed coat development and other processes (Gonzalez, et al., 2009; Debeaujon, et al., 2003; Ishida, et al., 2007). and can act as an scaffold protein along with TTG1 (WD40) in many cases to recruit different proteins together into a complex. For example, in the regulation of proanthocyanidin biosynthesis, it interacts with TT2 (MYB) and TT8 (bHLH)(Debeaujon, et al., 2003; Marles, et al., 2003), while in mucilage biosynthesis, it recruits MYB5 and EGL3/TT8 (Gonzalez, et al., 2009) and in trichome development interacts with GL1 and GL3/EGL3 (Zhao, et al., 2008). Therefore, it seems reasonable to postulate that TTG2 could be acting as a scaffold protein and may bring MYB75-KNAT7 complex together and regulate secondary cell wall formation. In similar fashion it may recruit only KNAT7 together with different mucilage-specific regulators like MYB5 (Li, et al., 2009b; Gonzalez, et al., 2009)(Figure 4.6B) to form a mucilage regulatory complex. The *TTG2* and *KNAT7* genetic interaction (or may be protein interaction?) could be a potential candidate to explain how the switch between two closely related but still diverse mucilage and secondary cell wall biosynthesis pathways takes place and what other factors are involved. In addition to MYB75 and KNAT7, other transcription factors regulating secondary cell wall formation in different tissue types (Zhong and Ye, 2007; Zhou, et al., 2009; Zhong, et al., 2008; Zhong and Ye, 2009) are also potential candidates that might be responsible for this regulation in other tissues and are part of the seed coat development network. Similarity of *myb75 knat7* in mutant seed coat phenotypes to that of *myb75* and the physical interaction between MYB75 and KNAT7 may indicate a conserved and specific regulatory complex. This interaction may be specific for secondary cell wall regulatory machinery similar to what was observed in inflorescence stems earlier (Chapter 3). Transcription factors show protein-protein interaction and form a plausible premise that in different tissue types these interactions may bind in different complexes to regulate similar phenotypes depending on spatial and temporal expression of different TFs. Conserved regulatory roles but different interacting partners of the transcription factor complexes have been shown earlier in invertebrate and mammalian system (Dallman, et al., 2004). In plants, it is now well established that some protein interactions are conserved in different tissue types during transcriptional regulation but also require many other interacting protein partners to provide tissue specificity. For example, in Arabidopsis, the WD

protein TTG1 interacts with the bHLH protein TT8 and MYB protein TT2 (Lepiniec, et al., 2006; Baudry, et al., 2004; Nesi, et al., 2001) to regulate transcriptional activation of flavonoid biosynthesis in mature seeds. However, in seedlings, TTG1 interaction with TT8 is conserved but in this instance interaction with MYB75 regulates flavonoid biosynthetic genes (Gonzalez, et al., 2008). This indicates the role of a conserved interaction (TTG1-TT8) in regulating the flavonoid pathway in different tissue types (seedlings and seeds) and also a role for different interacting partners (TT2 and MYB75) to give specificity in different tissue types. Similarly, VND7 (a NAC family transcription factor) interacts with different VND family proteins (VND2 to VND5) in a conserved manner to regulate differentiation of vessels in Arabidopsis but the specificity of the differentiation in roots and shoots is provided by interaction with other regulatory proteins through protein-protein interactions (Yamaguchi, et al., 2008). On a similar note, a regulatory role of the observed MYB75-KNAT interaction in secondary cell wall formation can be attributed to different partners in two different tissue type i.e. inflorescence stem and seed coat. The conserved functional role of MYB75-KNAT7 interaction in different tissue types may be dependent on different set of interacting proteins as postulated based on Y2H and BiFC data presented in this work (Figure 4.5) on seed coat and earlier in inflorescence stem (Chapter 3). In addition, the interaction of KNAT7 with a known mucilage biosynthesis regulator MYB5 (Li, et al., 2009b; Gonzalez, et al., 2009) could form a potential complex specific for a mucilage sub branch regulation in the overall seed epidermal regulation by transcription factors (Figure 4.6B).

It seems from the data investigated in this and earlier work (Chapter 2; Chapter 3; Brown, et al., 2005; Li, 2009) that MYB75 and KNAT7 not only have broader function as regulators of many different physiological functions, their biological role as regulators of secondary cell wall formation may be conserved in different tissue types (Chapter 2; this work). These two transcription factors show protein-protein interaction which seems to be functionally conserved in different tissue types (Chapter 3; Figure 4.5) but this interaction has other tissue-specific protein interacting partners as well (Chapter 3; Figure 4.5; Li, 2009). Future work will have to integrate these tissue specific interactions with a conserved interaction to look for the diversity provided by transcription factors in Arabidopsis.

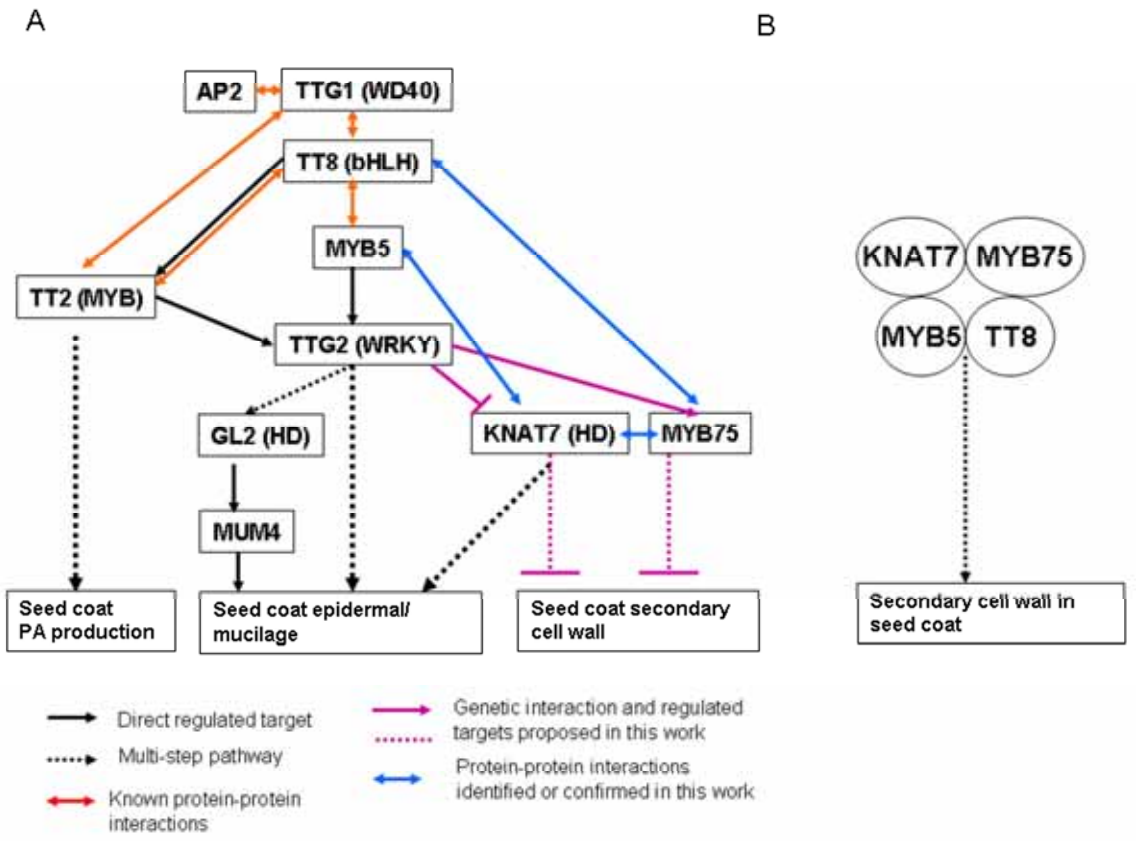


Figure 4.6 (A) A proposed regulatory network for seed coat epidermal differentiation control and with focus on secondary cell wall formation/mucilage biosynthesis. Solid arrows indicate direct regulation of targets. Dashed arrows indicate a multi-step differentiation pathway. Red colored bi-directional arrows indicate known protein-protein interactions. Blue colored bi-directional arrows indicate protein-protein interactions identified or confirmed in this work. Purple colored arrows indicate the identified regulatory targets. Note the negative regulation of secondary cell wall formation in seed coat by MYB75-KNAT7 interaction and specificity of MYB75 in regulating secondary cell wall formation in seed coat. (B) Proposed protein complex of transcription factors regulating secondary cell wall formation in the Arabidopsis seed coat.

Chapter 5. Conclusions and future directions

5.1 SUMMARY OF THE RESULTS

In this thesis, I analyzed the role of the *Arabidopsis thaliana* MYB75 transcription factor (TF) in regulation of secondary cell wall formation, with a particular focus on its interaction, biochemically and genetically, with other gene products. Interactions between MYB75 and a second transcription factor, KNAT7, were studied in detail and the biological significance of this association in regulating secondary cell wall formation was examined in two distinct tissues, the inflorescence stem and the seed coat.

In Chapter 2, I showed a unique contribution of MYB75 to secondary cell wall biogenesis through its influence on lignin deposition, specifically in the inflorescence stem. MYB75 is a known regulator of anthocyanin deposition through its influence on the phenylpropanoid pathway in Arabidopsis, but I asked how this regulatory function might have an impact on other aspects of carbon metabolism, including formation of secondary cell walls. The expression pattern and activation properties of MYB75 were studied in detail, which enabled me to conclude that this TF acts as a repressor of the lignin branch of the phenylpropanoid pathway and that it contributes to the regulation of secondary cell wall formation in the Arabidopsis stem. In agreement with suggestions from earlier studies that MYB75 might play a broader role in cellular metabolism, transcript profiles from the inflorescence stems of a MYB75 loss-of-function mutant revealed a marked up-regulation in the expression of a suite of genes associated with lignin biosynthesis and cellulose deposition, as well as those encoding cell wall modifying, photosynthesis and carbon assimilation-related proteins. This pattern is consistent with a model in which MYB75 is helping integrate the metabolic flux through the lignin, flavonoid and polysaccharide pathways in the Arabidopsis inflorescence stem.

Protein-protein interactions among transcription factors can lead to functional complex formation. In Chapter 3, it was shown that MYB75 (MYB)–KNAT7 (KNOX) physically interact and this interaction may form a complex to regulate secondary cell wall formation in Arabidopsis

inflorescence stem. In this part of the work, a group of known and potential secondary cell wall formation-regulating transcription factors in Arabidopsis were analyzed to detect any physical interactions among the group members. Many positive associations were observed and confirmed. Based on the evidence for the involvement of MYB75 and KNAT7 in secondary cell wall formation in Arabidopsis stems, their biological role was explored in more detail through analysis of single and double loss-of-function mutants. This work showed that MYB75 and KNAT7 might act in the same pathway, perhaps as the components of a putative multi-protein complex. This work not only revealed a novel physical interaction between KNOX and MYB TF families, but also emphasized the broad interactive capacity of TFs to assemble into differential complexes, thereby enhancing the functional diversity of the finite number of TFs in Arabidopsis.

In Chapter 4, functional conservation of the biological role(s) of MYB75 and KNAT7 in regulating secondary cell wall formation in Arabidopsis was demonstrated by examination of this process in another tissue, the seed coat. Genetic interactions of *MYB75* and *KNAT7* with other known transcription factors involved in seed coat development were defined, and a role for these two TFs in regulating radial wall thickness in the seed coat epidermal cells was revealed. Unlike KNAT7, MYB75 was shown to be a specific regulator of secondary cell wall formation with no apparent role in mucilage biosynthesis. Protein-protein interactions of MYB75 and KNAT7 with other transcription factors known to be involved in regulation of seed coat development were assessed and these results led to the proposal that a specific protein complex might form part of the secondary cell wall biosynthesis regulatory network in the seed coat epidermis.

5.2 FUTURE DIRECTIONS

5.2.1 Defining functional- and cell- specificity of MYB75

Functional specificity of MYB75

The data in my dissertation suggest that a loss of *MYB75* function results in the channeling of carbon toward the lignin pathway, generating increased lignin accumulation in secondary cell walls, whereas constitutive over-expression of *MYB75* leads to activation of anthocyanin biosynthesis-related genes and enhanced carbon flow into the flavonoid pathway. However, it remains possible that the visible increase in anthocyanin production associated with over-

expression of MYB75 does not reflect the endogenous function of MYB75 as suggested earlier (Gonzalez, et al., 2008; Borevitz, et al., 2000). Over-expression of transcription factors can potentially generate artefactual, pleiotropic phenotypes and therefore secondary cell wall regulation could be the main function of this transcription factor. More precisely defining the role of MYB75 and identifying its core function is an important objective for future work. To confirm if MYB75 is dedicated to secondary cell wall formation in the inflorescence stem and the associated phenotype not revealed by *MYB75(o/x)* in interfascicular fibers but shown in vessels in this work, overexpressing it under control of a promoter specific for relevant tissues should disclose the phenotype governed by MYB75. Interfascicular fibers, xylary fibers and vessels are the main areas of deposition of secondary cell wall in Arabidopsis inflorescence stem therefore one approach could be using a xylem-specific promoter such as the *4CL1* promoter to overexpress *MYB75*. Alternatively, other xylem marker gene promoters (Zhao, et al., 2005) like cambium/xylem marker *EXPA9* (Gray-Mitsumune, et al., 2004), and secondary cell wall-specific cellulose synthesis genes *CesA8* and *CesA4*, (Taylor, et al., 2003) could be used to overexpress MYB75.This could be a promising strategy to confirm the secondary cell wall phenotype associated with this TF. Further, it will also be worth to look and compare the regulatory role imparted by this transcription factor in anthocyanin biosynthesis when using such secondary cell wall-specific promoters.

Functional role of MYB75 homologs in secondary cell wall formation

Function of many TFs regulating a specific biosynthetic pathway may also be conserved for other pathways especially if the individual components of these interactions show genetic redundancy. This redundancy among the members of a big family of transcription factors like MYB is very common. Several MYBs along with MYB75 have been shown earlier to be involved in activation of anthocyanin biosynthesis (Gonzalez, et al., 2008). These other MYB75-related anthocyanin regulators may also have role in secondary cell wall formation in Arabidopsis. MYB75 belongs to a subfamily that includes MYB113 (At1g66370), MYB114 (At1g66380) and PAP2 (At1g66390), all of which have high sequence similarity and a conserved anthocyanin biosynthesis regulating role. It is possible that all of these members of the subfamily also have negative regulatory role in secondary cell wall formation similar, as demonstrated by MYB75. Therefore, to understand the complete anthocyanin pathway regulation by these MYBs, multiple mutants might be required (Gonzalez, et al., 2008). Recombination may be impractical in this

case as these genes are tightly linked tandemly on chromosome 1. To create plants that are deficient in all four MYB gene functions, an RNAi approach could be adopted. A similar approach was used by Gonzalez et al., 2008. These plants could be analyzed for phenotypes associated with secondary cell wall formation. In some cases, specific RNAi targeting of small regions that potentially distinguish closely-related sets of genes can be difficult, so this strategy might not be successful. As an alternative, it should be possible to use an artificial microRNA (miRNA) strategy. These single-stranded 21-mer RNAs are processed in a manner that one single stable small RNA is generated and specifically silences its intended target gene(s), either single or multiple related target genes (Schwab, et al., 2010)(http://wmd2.weigelworld.org/cgi-bin/mirnatools.pl?page=1).

Cell-specificity of MYB75

Cell-specificity of MYB75 in regulating secondary cell wall formation will be an important question to address in future works. Results from Chapter 2 indicate the role of this TF in regulating secondary cell wall formation in the inflorescence stem of Arabidopsis. However, the secondary cell wall deposition phenotype in the loss-of-function mutant of MYB75 was specific to interfascicular fiber cells and vessels appeared to be unaffected (Figure 2.4). In contrast, vessels in the MYB75-overexpression mutant showed a decrease in the cell wall thickness. This may indicate a very spatial and dose-specific regulation of secondary cell wall deposition by MYB75. As noted in Chapter 3, and in earlier work (Li, 2009), loss of KNAT7 also has an opposite effect on secondary cell wall thickening in the vessels as compared to interfascicular fibers. These patterns of cell wall modification in two different adjacent cells give rise to an interesting question. Do these TFs have different roles in different cell types? Many different approaches can be adopted to answer this question. One possible strategy would be to separately analyze *MYB75* and *KNAT7* gene expression in developing interfascicular fibers and vessels of the Arabidopsis stem by laser capture microdissection (LCM). These results can be compared with the tissue-specific expression profiles for similar LCM-isolated tissue samples from *myb75* and *knat7* mutants. Chemical microanalysis (lignin, sugars etc.) relative to wild type cells on these LCM separated samples can also be performed, as described earlier (Ruel, et al., 2009). This would provide further insight to the two different phenotypes seen in *myb75* and *knat7* vessels and interfascicular fibers.

The tissue-specific expression profiling could be complemented by immunolocalization of KNAT7 and MYB75 in inflorescence stem sections using mono-specific antibodies or antibodies directed at epitope-tagged versions of the proteins. This would enable me to detect any differential patterns of distribution of the proteins in interfascicular fibers, vessels and xylary fibers in the Arabidopsis stem.

5.2.2 Identifying downstream targets of MYB75 and KNAT7

Characterization of a double mutant (Chapter 3) to look for genetic interactions between MYB75 and KNAT7 was part of this work. This work indicated that these TFs may have roles in regulating broader carbon redistribution in Arabidopsis stems in addition to role in secondary cell wall formation and lignification. In addition to these roles of MYB75 and KNAT7, it is important to know the farther downstream targets that these transcription factors might control, individually and by interacting with each other. Global gene profiling using *myb75* stems in Chapter 2 showed differential expression of a set of downstream target genes. However, this was the overall effect and it was not possible to separate direct regulatory targets from indirect regulation. To more precisely define the function of MYB75 or KNAT7, it is important to look for specific and direct targets. One approach to identification of such specific downstream targets could be to use inducible systems such as the TF(s)-glucocorticoid receptor (GR) system, where transgene expression is responsive to application of a chemical inducer such as dexamethasone (DEX). This requires fusion of MYB75 or KNAT7 with GR under control of the native promoter or 35S promoter and transforming these constructs into either *myb75* or *knat7* mutant plants. Treatment of these transgenic lines with DEX is expected to mobilize MYB75-GR or KNAT7-GR from the cytoplasm to the nucleus, activating either MYB75 or KNAT7 target genes. Microarray analysis of transcripts isolated from the stem samples could then be performed to identify tissue-specific downstream differentially expressed genes using control transgenic lines transformed with the empty vector containing GR alone.

Another interesting question to address is the identity of downstream targets of MYB75 and KNAT7 in the developing seed coat. Cellulose synthase 9 has been shown recently to be involved in secondary cell wall thickening in this tissue (Stork, et al., 2010). However, how CesA9 or other downstream enzymes in this secondary cell wall biosynthetic pathway in seed

coat are regulated is unknown and there is a possibility that either MYB75 or KNAT7 help regulate expression of secondary cell wall- associated enzymes in the seed coat as they do in the inflorescence stem (Chapter 3). Gene profile studies on the 11 DPA seed coat stage in *myb75* and *knat7* plants could potentially identify the global gene targets affected by the loss-of-function of these genes in the seed coat. It will be useful to compare such datasets with the gene expression profile obtained in the microarray for *myb75* inflorescence stem in this work (Table 2.6, Chapter 2) to highlight the differences in the tissue specificity and role of these TFs in these two different tissues. This will not only be helpful to explain the phenotype related to secondary cell wall and or mucilage that was seen in Chapter 4, but will also help in understanding the position of these TFs in the genetic network more clearly.

As mentioned earlier, it is not clear with a non-targeted microarray whether the observed gene expression changes are due to direct or indirect regulation. This makes it challenging and time-consuming to define the primary gene targets. The localization of genomic TF binding sites through the immunoprecipitation of a TF, can be obtained using chromatin immunoprecipitation (ChIP). It is a very useful tool to identify regulatory (promoter) regions directly bound by a TF in the context of the chromatin structure. ChIP dissects the spatial and temporal interactions of chromatin and its associated factors. For determining the downstream direct targets of MYB75 and /or KNAT7, KNAT7-, MYB75- specific antibody or the antibodies against the tags attached to these TFs (e.g. His, Myc or HA) that recognize these protein or their modification can be used to find out their relative abundance at one or more locations in the genome. Combining ChIP and micro-array technology (ChIP on chip) could allow genome-wide analysis of these transcription factors distributions. Immunoprecipitated chromatin could be then combined with a DNA microchip. In this way a complete genome map for these transcription factors can be assembled (Bernstein, et al., 2002). The tissue specific targets can be identified using chromatin from a specific tissue, in this case separately from inflorescence stem and seed coat of Arabidopsis.

5.2.3 Characterizing broader regulatory roles of MYB75 in carbon redistribution and maintaining carbon-nitrogen sink

The transcript profiles (Table 2.5 and Table 2.6) from the *myb75-1* inflorescence stems reflect possible carbon flux re-distribution within the branches of phenylpropanoid metabolism, as well

as into other metabolic pathways. These patterns provide an indication of a broader role for this transcription factor in photosynthesis, with significant changes in genes associated specifically with photosynthetic apparatus (Table 2.6). More physiological experimental evidence such as measurements of photosynthetic efficiency in *myb75* plants compared to WT would help define a possible role for MYB75 as a broader regulator of the overall carbon flux in Arabidopsis. The net photosynthetic rate of *myb75* leaves relative to WT leaves could be measured using an infra-red gas analyzer, which assesses the net exchange of CO_2 between leaves and atmosphere by monitoring the rate of CO_2 concentration change over short time intervals. In addition, comparison of chlorophyll and carotenoid content in the loss-of-function mutant and WT could be performed. Change in chlorophyll content could reflect alterations in the capacity of the light harvesting complexes of photosystem II (PS II). Possible changes in the overall activity of PSII in *myb75* leaves as indicated by the microarray profiling (Table 2.5), could potentially be detected by measuring chlorophyll fluorescence (quantum yield of PSII) using a chlorophyll fluorometer.

Gene profiling of *myb75* stems also showed that some genes involved in cell wall modification were upregulated (Table 2.5), and some of these, such as AGP4, have not been demonstrated to play a role in secondary cell wall formation in Arabidopsis. Since an arabinogalactan protein was shown to be associated with secondary cell wall formation in xylem of loblolly pine (Zhang, et al., 2003), functional characterization of *AGP4* (At5g10430) by reverse genetics and biochemical approaches could allow us to identify the link between transcription factor regulation and arabinogalactan protein function. Similarly, a few previously uncharacterized glycosyl hydrolases were shown to be up-regulated in the microarray profiling in this work (At1g45130 and At5g26000). Cell wall glycosyl hydrolases are thought to degrade cell walls to release sugars under carbon starvation conditions and, thereby, remobilize sugars to other sinks (Lee, et al., 2007). In light of the proposed role of MYB75 as a mediator of carbon redistribution, characterization of these hydrolases could provide important new insights into such a process.

The evidence in this work that MYB75 may play a broader role in carbon re-distribution opens up other questions. Other than carbon sink(s), nitrogen is often a limiting nutrient in the environment and there is a limited supply of nitrogen in plants. Plants have therefore evolved efficient intracellular mechanisms to capture and reassimilate inorganic nitrogen that is liberated during metabolic processes. Therefore, cellular carbon (C) and nitrogen (N) metabolism must be

tightly coordinated to sustain optimal growth and development for plants and other cellular organisms (Zheng, 2009). High carbon and low nitrogen ratios are maintained by many plants (Lea, et al., 2007; Zheng, 2009). If MYB75 has a role in regulating broader carbon distribution as indicated in this thesis, then there is a possibility that it also has a role in regulating some aspects of nitrogen metabolism. Therefore, maintenance of ratio of carbon to nitrogen (C/N) can be proposed to be mediated by this transcription factor. Secondary cell walls represent immense carbon sink in plants and lignin is an important constituent of these cell wall. Deamination of phenylalanine by the entry-point enzyme, phenylalanine ammonia-lyase (PAL), is a key step that commits the phenylalanine carbon skeleton to the phenylpropanoid pathway which generates lignin (Singh, et al., 1998). The PAL-catalyzed reaction releases ammonia and, therefore, lignification of cell walls during active secondary cell wall synthesis could create a significant nitrogen deficiency if lignifying cells and/or their neighbors did not possess efficient nitrogen recovery mechanisms that couple nitrogen recycling and lignification in both time and space. This coordinated metabolic pattern can be predicted to be reflected in transcriptional co-regulation patterns in which specific TFs simultaneously regulate both lignin biosynthesis and enzymes involved in nitrogen recovery. It was earlier reported that MYB proteins might play important role in the coordinated control of both of these processes (Gomez-Maldonado, et al., 2004). Microarray profiling of Arabidopsis plants grown under nitrogen deficiency conditions has revealed that MYB75 is highly up-regulated under this condition (Lea, et al., 2007) while reverse genetics studies have revealed that this TF negatively regulate lignin biosynthesis (Chapter 2). Therefore, MYB75 has the potential to act as a regulator that helps maintain optimal C/N ratios in the Arabidopsis stems. To further explore this model, *myb75* mutants can be analysed for changes in the expression profiles of key genes involved in nitrogen assimilation. The constitutive expression levels of genes such as glutamine synthetase (GS), phosphoenolpyruvate carboxylase (PEPC), glutamate synthase (GOGAT) and nitrate reductase (NR) can be measured using real time qRT-PCR and compared with levels observed in wild-type plants, both under the conditions of nitrogen sufficiency and deficiency. If these results are promising, one could consider conducting assays for the corresponding protein levels (commercial PEPC antibody is available, for example) and enzyme activities (Yanagisawa, et al., 2004). Metabolite analyses could also be carried out on pooled Arabidopsis plants to measure the

levels of key carbon (glucose, sucrose, fructose, xylose, oxaloacetate, malate, citrate and α ketoglutarate) and nitrogen (nitrate, nitrite, and all amino acids) substrates.

5.2.4 Elucidating additional interacting partners of MYB75 and KNAT7 *in vitro* and *in planta*

Transcription factor complexes are composed of more than two interacting proteins. I was able to identify an interaction between two transcription factors (MYB75 and KNAT7) and its potential role in secondary cell wall formation. Identifying other partners of this complex is an important question to reveal the complete biological significance of the regulatory complex involved in this cell wall biosynthesis regulation. Yeast two-hybrid (Y2H) library screening could be used to identify additional interactors. Tissue specific (inflorescence stem and seed coat) and Gateway®-compatible Y2H library could be constructed and can be used to identify additional interaction partners for the candidate transcription factors (KNAT7 and MYB75) by using these TFs as baits. This approach can identify putative members of complexes regulating secondary cell wall formation in Arabidopsis stems and seeds.

The major limitation of the yeast two-hybrid technique is that it does not fully mimic the physiological conditions under which plant proteins normally function. This artifact can result in the failure to identify specific protein-protein interactions, or sometimes, to encounter a high false discovery rate because of promiscuous and unreal interactions. It will therefore be necessary to complement the Y2H approach with the use of tandem affinity purification of protein complexes recovered directly from transgenic Arabidopsis plants. By affinity tagging each of the candidate transcription factors and using these chimeric constructs as *in vivo* baits, it is possible to recover physiologically relevant complexes and to determine the identity of the individual components of the purified multi-protein complex using mass spectrometry (MS). Affinity tagging in this fashion has the added advantage that it can potentially identify proteins that participate in forming a complex with the candidate, even if they are not directly bound by this TF (i.e. secondary interactors). This work has already been initiated and needs to be completed to characterize the complexes. MYB75 and KNAT7 have been cloned into specialized Gateway® compatible vectors (Rubio, et al., 2005) that incorporate a Tandem Affinity Purification (TAP) tag into each protein when expressed *in planta*. Arabidopsis plants have been transformed with these TAP-tagged genes and lines with good integration and expression pattern have been

selected. For protein complex isolation, both nuclear-enriched and tissue specific (from inflorescence stem and seed coat) and total protein extraction approaches can be used in future work. After allowing the TAP-tagged protein to bind to the affinity matrix, and washing away proteins that interact nonspecifically with the chimeric bait protein and/or matrix, the remaining protein complexes can be eluted, fractionated by 1-D PAGE and/or directly analyzed by mass spectrometry. This in future will provide *in vivo* data from TAP-tagged plants about the MYB75-KNAT7 interactions. We will see Y2H and BiFC identified interacting partners (Chapter 2) in the proteins brought down with the TAP tag, if these are significant interactions *in planta*. This technique being able to pull down native interactors from the plants itself may provide more promising interacting candidates for future biological studies. If this technique yields promising results, future analyses can focus on native interactions by using mutant backgrounds. In this strategy, the expression of the endogenous protein is suppressed. Expression of the protein tag in a loss-of-function background may help to avoid competition between the ectopically expressed and endogenous proteins, thereby facilitating recovery of true native complexes.

Using these approaches (Y2H library analysis, and TAP-tagging) it is possible to identify new interacting protein candidates, both in inflorescence stem and seed coat that will require further validation and characterization.

5.2.5 Establishing distinction between mucilage and secondary cell wall formation regulation

In Chapter 4, I was able to demonstrate that MYB75 may be a very specific TF for secondary cell wall formation but KNAT7 has a role in both secondary cell wall formation and mucilage biosynthesis in Arabidopsis seed coat. In this context, it is noteworthy that the potential of MYB75 and KNAT7 to form different protein complexes and the complex transcriptional regulation indicate different active feedbacks that might establish different networks and activities of these TFs in different cells/tissue types. However, to further strengthen the evidences that I obtained for MYB75 distinct role in secondary cell wall formation in seed coat, future investigations will be required. Since *myb75* and *knat7* have altered gene expression in hemicellulose- and cellulose– associated genes (Chapter 3, Figure 3.11) in stems, it is possible that similar expression is also conserved in the seed coat and is a promising experiment to

continue in the future. In particular for *knat7*, it is possible that these postulated changes in cellulose- and hemicellulose-specific genes resulting in changes in mucilage composition (changes in pectin network). Secondary cell wall-specific gene expression profile in seed coat stage (mature) in different mutants including *myb75* and *knat7* is required to look for further answers. In addition, expression profile of seed coat specific enzymes involved in these two biosynthesis pathways is required. For example, CesA9 that was recently shown to be seed coat-specific cellulose synthase (Stork, et al., 2010), *MUM2* encoding a β-galactosidase that is required to modify pectin structure in the mucilage (Dean, et al., 2007) and *MUM4* that encodes a UDP-L-rhamnose synthase, required for the mucilage pectin biosynthesis (Western, et al., 2004; Usadel, et al., 2004; Oka, et al., 2007). Further, chemical analysis of mucilage (pectin), total sugars (Cellulose and hemicellulose), lignin and cell wall-associated proteins in *myb75* and *knat7* mutant seeds relative to WT will cast more light on the role of these transcription factors in regulating these biochemical pathways.

5.2.6 Testing the proposed protein complex model for its role in secondary cell wall formation in seed coat

To understand the broader specificity of the proposed complex formation for the MYB75 transcription factor in the seed coat (Figure 4.6B), yeast three-hybrid (Y3H) assay could be performed as previously described by Immink et al, (2009). In short, for this purpose, all the TFs that are found to interact in yeast two-hybrid assay and BiFC assays could be tested in an appropriate yeast strain by expressing one of the two partners as a fusion with the activation domain (AD) of the yeast GAL4 transcription factor, and the second TF fused to a nuclear localization signal only (Ciannamea, et al., 2006). Subsequently, these yeast clones can be either screened against the third interacting TF fused to the GAL4 binding domain (BD) (de Folter, et al., 2005) or also to an available collection of TFs in a seed coat-specific library fused to the GAL4 binding domain (BD) in the same yeast strain to identify other and novel interacting partners of the protein complex.

It has been reported earlier that MYB5 is involved in mucilage biosynthesis in the seed coat (Li, et al., 2009b; Gonzalez, et al., 2009). MYB5 interacted with KNAT7 in this study but the question whether this interaction has a role in secondary cell wall formation or in mucilage

biosynthesis remains unanswered. It is possible that KNAT7 plays a dual role in regulating both mucilage and secondary cell wall formation in seed coat but involves different complexes. There is also a possibility of a ternary complex consisting of TT8-KNAT7-MYB5 (all with known role in mucilage biosynthesis) in regulating mucilage biosynthesis and not secondary cell wall in seed coat in addition to the complex proposed (Figure 4.6B) for secondary cell wall formation. Generating double mutants between KNAT7-MYB5, triple mutant (KNAT7-MYB5-TT8) and quadruple mutants between MYB75-KNAT7-TT8-MYB5 will help to understand and confirm the role of protein complex in regulating different aspects of seed coat development.

In addition, the role of TTG2 is not very clear from this work in secondary cell wall formation. Elucidating protein-protein interaction between KNAT7-TTG2-MYB75 could also cast some light on the regulatory role of TTG2 in generating different downstream complexes in the network (Figure 4.6A). Characterization of *ttg2* phenotype with respect to secondary cell wall formation in seed coat and stems may clarify its conserved role in seed coat and regulation of inflorescence stem secondary cell wall formation.

5.2.7 Characterization of additional and identified protein-protein interactions by Y2H in secondary cell wall regulation in Arabidopsis

Many positive yeast 2-hybrid interactions were observed among the potential transcription factors tested (Chapter 3) belonging to different families. I characterized MYB75-KNAT7 interaction in detail in this thesis (Chapter 3 and Chapter 4) for its role in secondary cell wall formation in Arabidopsis stem. However, there were other positive protein-protein interactions observed from yeast in my work (AP2-family TF interaction with bZIP47/TGA1 and MYB63 interaction with TT8) which might have similar importance in forming regulatory complexes involved in secondary cell wall formation. Among these interaction TFs, MYB63 (Zhou, et al., 2009) has already been demonstrated to be a regulator of secondary wall formation in inflorescence stem, TT8 is a known seed coat regulator and have role in mucilage and proanthocyanidin regulation (Nesi, et al., 2001; Nesi, et al., 2000). Hence, a MYB63-TT8 complex may be an important regulator in seed coat secondary wall formation. AP2/EREBP family protein has been shown earlier to be involved in regulation of seed coat development (APETALA2, (Western, et al., 2000; Ohto, et al., 2009) as well as in other floral regulation

(Ilegems, et al., 2010; Kunst, et al., 1989; Irish and Sussex, 1990). To my knowledge, no member of this TF family has been shown to play a role in any aspect of cell wall regulation. Therefore, the potential cell wall regulatory candidate AP2 family member (At5g07580) showing protein-protein interaction in my work is a promising candidate to analyze. A role for the TGA family of bZIP TFs in mediating systemic acquired resistance and *PR* gene expression is known (Pontier, et al., 2001; Fan and Dong, 2002; Niggeweg, et al., 2000). There is no report of involvement of these factors in secondary cell wall formation or any related mechanism. Therefore, the interaction between AP2-family TF with bZIP47/TGA1 detected in my work (Figure 3.1) may also be involved in forming a secondary cell wall regulatory complex. These transcription factors also represent novel candidates to characterize for their role in secondary cell wall formation individually. Characterization of their loss-of function mutants, generation of different double mutants, histochemical and biochemical analysis for secondary cell wall traits and identifying other potential interactors could reveal the role of these TF complexes in future work.

Bibliography

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell **15:** 63-78

An YQ, McDowell JM, Huang S, McKinney EC, Chambliss S, Meagher RB (1996) Strong, constitutive expression of the Arabidopsis ACT2/ACT8 actin subclass in vegetative tissues. Plant J **10:** 107-121

Ban Y, Honda C, Hatsuyama Y, Igarashi M, Bessho H, Moriguchi T (2007) Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red coloration in apple skin. Plant Cell Physiol **48:** 958-970

Baudry A, Heim MA, Dubreucq B, Caboche M, Weisshaar B, Lepiniec L (2004) TT2, TT8, and TTG1 synergistically specify the expression of BANYULS and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. Plant J **39:** 366-380

Beeckman T, De Rycke R, Viane R, Inzé D (2000) Histological study of seed coat development in *Arabidopsis thaliana*. J Plant Res **113:** 139-148

Bellaoui M, Pidkowich MS, Samach A, Kushalappa K, Kohalmi SE, Modrusan Z, Crosby WL, Haughn GW (2001) The Arabidopsis BELL1 and KNOX TALE homeodomain proteins interact through a domain conserved between plants and animals. Plant Cell **13:** 2455-2470

Belles-Boix E, Hamant O, Witiak SM, Morin H, Traas J, Pautot V (2006) KNAT6: an Arabidopsis homeobox gene involved in meristem activity and organ separation. Plant Cell **18:** 1900-1907

Bernhardt C, Lee MM, Gonzalez A, Zhang F, Lloyd A, Schiefelbein J (2003) The bHLH genes GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) specify epidermal cell fate in the Arabidopsis root. Development **130:** 6431-6439

Bernstein BE, Humphrey EL, Erlich RL, Schneider R, Bouman P, Liu JS, Kouzarides T, Schreiber SL (2002) Methylation of histone H3 Lys 4 in coding regions of active genes. Proc Natl Acad Sci U S A **99:** 8695-8700

Bertolino E, Reimund B, Wildt-Perinic D, Clerc RG (1995) A novel homeobox protein which recognizes a TGT core and functionally interferes with a retinoid-responsive motif. J Biol Chem **270:** 31178-31188

Bhatt AM, Etchells JP, Canales C, Lagodienko A, Dickinson H (2004) VAAMANA--a BEL1-like homeodomain protein, interacts with KNOX proteins BP and STM and regulates inflorescence stem growth in Arabidopsis. Gene **328:** 103-111

Birnbaum K, Shasha DE, Wang JY, Jung JW, Lambert GM, Galbraith DW, Benfey PN (2003) A gene expression map of the Arabidopsis root. Science **302:** 1956-1960

Bloom AJ, ChapinIII FS, Mooney HA (1985) Resource limitation in plants-an economic analogy. Annual Review of Ecology and Systematics **16:** 363-392

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol **54:** 519-546

Bomal C, Bedon F, Caron S, Mansfield SD, Levasseur C, Cooke JE, Blais S, Tremblay L, Morency MJ, Pavy N, Grima-Pettenati J, Seguin A, Mackay J (2008) Involvement of *Pinus taeda* MYB1 and MYB8 in phenylpropanoid metabolism and secondary cell wall biogenesis: a comparative in planta analysis. J Exp Bot **59** (14)**:** 3925-39

Bonke M, Thitamadee S, Mahonen AP, Hauser MT, Helariutta Y (2003) APL regulates vascular tissue identity in Arabidopsis. Nature **426:** 181-186

Borevitz JO, Xia Y, Blount J, Dixon RA, Lamb C (2000) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell **12:** 2383-2394

Braun EL, Grotewold E (1999) Newly discovered plant c-myb-like genes rewrite the evolution of the plant myb gene family. Plant Physiol **121:** 21-24

Broun P (2005) Transcriptional control of flavonoid biosynthesis: a complex network of conserved regulators involved in multiple aspects of differentiation in Arabidopsis. Curr Opin Plant Biol **8:** 272-279

Brown DM, Zeef LA, Ellis J, Goodacre R, Turner SR (2005) Identification of novel genes in Arabidopsis involved in secondary cell wall formation using expression profiling and reverse genetics. Plant Cell **17:** 2281-2295

Brown DM, Zhang Z, Stephens E, Dupree P, Turner SR (2009) Characterization of IRX10 and IRX10-like reveals an essential role in glucuronoxylan biosynthesis in Arabidopsis. Plant J **57:** 732-746

Burglin TR (1997) Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. Nucleic Acids Res **25:** 4173-4180

Carpita N, Tierney M, Campbell M (2001) Molecular biology of the plant cell wall: searching for the genes that define structure, architecture and dynamics. Plant Mol Biol **47:** 1-5

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J **3:** 1-30

Chory J, Nagpal P, Peto CA (1991) Phenotypic and Genetic Analysis of det2, a New Mutant That Affects Light-Regulated Seedling Development in Arabidopsis. Plant Cell **3:** 445-459

Ciannamea S, Kaufmann K, Frau M, Tonaco IA, Petersen K, Nielsen KK, Angenent GC, Immink RG (2006) Protein interactions of MADS box transcription factors involved in flowering in *Lolium perenne*. J Exp Bot **57:** 3419-3431

Coleman HD, Park JY, Nair R, Chapple C, Mansfield SD (2008) RNAi-mediated suppression of p-coumaroyl-CoA 3'-hydroxylase in hybrid poplar impacts lignin deposition and soluble secondary metabolism. Proc Natl Acad Sci U S A **105:** 4501-4506

Coruzzi G, Bush DR (2001) Nitrogen and carbon nutrient and metabolite signaling in plants. Plant Physiol **125:** 61-64

Coruzzi GM, Zhou L (2001) Carbon and nitrogen sensing and signaling in plants: emerging 'matrix effects'. Curr Opin Plant Biol **4:** 247-253

Cosgrove DJ (2005) Growth of the plant cell wall. Nat Rev Mol Cell Biol **6:** 850-861

Cosgrove DJ (1996) Plant cell enlargement and the action of expansins. Bioessays **18:** 533-540

Dallman JE, Allopenna J, Bassett A, Travers A, Mandel G (2004) A conserved role but different partners for the transcriptional corepressor CoREST in fly and mammalian nervous system formation. J Neurosci **24:** 7186-7193

Darley CP, Forrester AM, McQueen-Mason SJ (2001) The molecular basis of plant cell wall extension. Plant Mol Biol **47:** 179-195

Davies KM, Schwinn KE (2003) Transcriptional regulation of secondary metabolism. Functional Plant Biology **30:** 913-925

de Folter S, Immink RG, Kieffer M, Parenicova L, Henz SR, Weigel D, Busscher M, Kooiker M, Colombo L, Kater MM, Davies B, Angenent GC (2005) Comprehensive interaction map of the Arabidopsis MADS Box transcription factors. Plant Cell **17:** 1424-1433

Dean GH, Zheng H, Tewari J, Huang J, Young DS, Hwang YT, Western TL, Carpita NC, McCann MC, Mansfield SD, Haughn GW (2007) The Arabidopsis MUM2 gene encodes a beta-galactosidase required for the production of seed coat mucilage with correct hydration properties. Plant Cell **19:** 4007-4021

Debeaujon I, Nesi N, Perez P, Devic M, Grandjean O, Caboche M, Lepiniec L (2003) Proanthocyanidin-accumulating cells in Arabidopsis testa: regulation of differentiation and role in seed development. Plant Cell **15:** 2514-2531

Demura T, Fukuda H (2007) Transcriptional regulation in wood formation. Trends Plant Sci **12:** 64-70

Desprez T, Juraniec M, Crowell EF, Jouy H, Pochylova Z, Parcy F, Hofte H, Gonneau M, Vernhettes S (2007) Organization of cellulose synthase complexes involved in primary cell wall synthesis in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A **104:** 15572-15577

Devaiah BN, Madhuvanthi R, Karthikeyan AS, Raghothama KG (2009) Phosphate starvation responses and gibberellic acid biosynthesis are regulated by the MYB62 transcription factor in Arabidopsis. Mol Plant **2:** 43-58

Diet A, Brunner S, Ringli C (2004) The enl mutants enhance the lrx1 root hair mutant phenotype of *Arabidopsis thaliana*. Plant Cell Physiol **45:** 734-741

Do CT, Pollet B, Thevenin J, Sibout R, Denoue D, Barriere Y, Lapierre C, Jouanin L (2007) Both caffeoyl Coenzyme A 3-O-methyltransferase 1 and caffeic acid O-methyltransferase 1 are involved in redundant functions for lignin, flavonoids and sinapoyl malate biosynthesis in Arabidopsis. Planta **226:** 1117-1129

Douglas CJ, Ehlting J (2005) *Arabidopsis thaliana* full genome longmer microarrays: a powerful gene discovery tool for agriculture and forestry. Transgenic Res **14:** 551-561

Douglas SJ, Chuck G, Dengler RE, Pelecanda L, Riggs CD (2002) KNAT1 and ERECTA regulate inflorescence architecture in Arabidopsis. Plant Cell **14:** 547-558

Du J, Groover A (2010) Transcriptional regulation of secondary growth and wood formation. J Integr Plant Biol **52:** 17-27

Dubos C, Le Gourrierec J, Baudry A, Huep G, Lanet E, Debeaujon I, Routaboul JM, Alboresi A, Weisshaar B, Lepiniec L (2008) MYBL2 is a new regulator of flavonoid biosynthesis in *Arabidopsis thaliana*. Plant J **55:** 940-953

Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L (2010) MYB transcription factors in Arabidopsis. Trends Plant Sci

Dubos C, Willment J, Huggins D, Grant GH, Campbell MM (2005) Kanamycin reveals the role played by glutamate receptors in shaping plant resource allocation. Plant J **43:** 348-355

Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS (2006) Gateway-compatible vectors for plant functional genomics and proteomics. Plant J **45:** 616-629

Ehlting J, Mattheus N, Aeschliman DS, Li E, Hamberger B, Cullis IF, Zhuang J, Kaneda M, Mansfield SD, Samuels L, Ritland K, Ellis BE, Bohlmann J, Douglas CJ (2005) Global transcript profiling of primary stems from *Arabidopsis thaliana* identifies candidate genes for missing links in lignin biosynthesis and transcriptional regulators of fiber differentiation. Plant J **42:** 618-640

Fan W, Dong X (2002) In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. Plant Cell **14:** 1377-1389

Fashena SJ, Serebriiskii I, Golemis EA (2000) The continued evolution of two-hybrid screening approaches in yeast: how to outwit different preys with different baits. Gene **250:** 1-14

Feng C, Andreasson E, Maslak A, Mock HP, Mattsson O, Mundy J (2004) Arabidopsis MYB68 in development and responses to environmental cues. Plant Science **167:** 1099-1107

Feng S, Wang Y, Yang S, Xu Y, Chen X (2010) Anthocyanin biosynthesis in pears is regulated by a R2R3-MYB transcription factor PyMYB10. Planta **232:** 245-255

Fornale S, Sonbol FM, Maes T, Capellades M, Puigdomenech P, Rigau J, Caparros-Ruiz D (2006) Down-regulation of the maize and *Arabidopsis thaliana* caffeic acid O-methyl-transferase genes by two new maize R2R3-MYB transcription factors. Plant Mol Biol **62:** 809-823

Frampton J, Gibson TJ, Ness SA, Doderlein G, Graf T (1991) Proposed structure for the DNA-binding domain of the Myb oncoprotein based on model building and mutational analysis. Protein Eng **4:** 891-901

Gardiner JC, Taylor NG, Turner SR (2003) Control of cellulose synthase complex localization in developing xylem. Plant Cell **15:** 1740-1748

Gehring WJ (1987) Homeo boxes in the study of development. Science **236:** 1245-1252

Gigolashvili T, Berger B, Mock HP, Muller C, Weisshaar B, Flugge UI (2007) The transcription factor HIG1/MYB51 regulates indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. Plant J **50:** 886-901

Gigolashvili T, Engqvist M, Yatusevich R, Muller C, Flugge UI (2008) HAG2/MYB76 and HAG3/MYB29 exert a specific and coordinated control on the regulation of aliphatic glucosinolate biosynthesis in *Arabidopsis thaliana*. New Phytol **177:** 627-642

Goff SA, Cone KC, Chandler VL (1992) Functional analysis of the transcriptional activator encoded by the maize B gene: evidence for a direct functional interaction between two classes of regulatory proteins. Genes Dev **6:** 864-875

Goicoechea M, Lacombe E, Legay S, Mihaljevic S, Rech P, Jauneau A, Lapierre C, Pollet B, Verhaegen D, Chaubet-Gigot N, Grima-Pettenati J (2005) EgMYB2, a new transcriptional activator from Eucalyptus xylem, regulates secondary cell wall formation and lignin biosynthesis. Plant J **43:** 553-567

Gomez-Maldonado J, Avila C, Torre F, Canas R, Canovas FM, Campbell MM (2004) Functional interactions between a glutamine synthetase promoter and MYB proteins. Plant J **39:** 513-526

Gomez-Mena C, Pineiro M, Franco-Zorrilla JM, Salinas J, Coupland G, Martinez-Zapater JM (2001) early bolting in short days: an Arabidopsis mutation that causes early flowering and partially suppresses the floral phenotype of leafy. Plant Cell **13:** 1011-1024

Gonzalez A, Mendenhall J, Huo Y, Lloyd A (2009) TTG1 complex MYBs, MYB5 and TT2, control outer seed coat differentiation. Dev Biol **325:** 412-421

Gonzalez A, Zhao M, Leavitt JM, Lloyd AM (2008) Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. Plant J **53:** 814-827

Grant EH, Fujino T, Beers EP, Brunner AM (2010) Characterization of NAC domain transcription factors implicated in control of vascular cell differentiation in Arabidopsis and Populus. Planta **232:** 337-352

Gray-Mitsumune M, Mellerowicz EJ, Abe H, Schrader J, Winzell A, Sterky F, Blomqvist K, McQueen-Mason S, Teeri TT, Sundberg B (2004) Expansins abundant in secondary xylem belong to subgroup A of the alpha-expansin gene family. Plant Physiol **135:** 1552-1564

Grotewold E, Sainz MB, Tagliani L, Hernandez JM, Bowen B, Chandler VL (2000) Identification of the residues in the Myb domain of maize C1 that specify the interaction with the bHLH cofactor R. Proc Natl Acad Sci U S A **97:** 13579-13584

Guimil S, Dunand C (2006) Patterning of Arabidopsis epidermal cells: epigenetic factors regulate the complex epidermal cell fate pathway. Trends Plant Sci **11:** 601-609

Hackbusch J, Richter K, Muller J, Salamini F, Uhrig JF (2005) A central role of *Arabidopsis thaliana* ovate family proteins in networking and subcellular localization of 3-aa loop extension homeodomain proteins. Proc Natl Acad Sci U S A **102:** 4908-4912

Hahlbrock,K.,Scheel,D. (1987) Biochemical responses to plant to pathogens. In I Chet, ed, Innovative approaches to plant disease control. Wiley, New york, pp 229-254

Hake S, Smith HM, Holtan H, Magnani E, Mele G, Ramirez J (2004) The role of knox genes in plant development. Annu Rev Cell Dev Biol **20:** 125-151

Haughn G, Chaudhury A (2005) Genetic analysis of seed coat development in Arabidopsis. Trends Plant Sci **10:** 472-477

Hay A, Tsiantis M (2010) KNOX genes: versatile regulators of plant development and diversity. Development **137:** 3153-3165

Heim MA, Jakoby M, Werber M, Martin C, Weisshaar B, Bailey PC (2003) The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. Mol Biol Evol **20:** 735-747

Humphreys JM, Chapple C (2002) Rewriting the lignin roadmap. Curr Opin Plant Biol **5:** 224-229

Ikeda M, Mitsuda N, Ohme-Takagi M (2009) Arabidopsis WUSCHEL is a bifunctional transcription factor that acts as a repressor in stem cell regulation and as an activator in floral patterning. Plant Cell **21:** 3493-3505

Ilegems M, Douet V, Meylan-Bettex M, Uyttewaal M, Brand L, Bowman JL, Stieger PA (2010) Interplay of auxin, KANADI and Class III HD-ZIP transcription factors in vascular tissue formation. Development **137:** 975-984

Immink RG, Tonaco IA, de Folter S, Shchennikova A, van Dijk AD, Busscher-Lange J, Borst JW, Angenent GC (2009) SEPALLATA3: the 'glue' for MADS box transcription factor complex formation. Genome Biol **10:** R24

Ingham PW (1998) trithorax and the regulation of homeotic gene expression in Drosophila: a historical perspective. Int J Dev Biol **42:** 423-429

Irish VF, Sussex IM (1990) Function of the *Apetala-1* gene during Arabidopsis floral development. Plant Cell **2:** 741-753

Ishida T, Hattori S, Sano R, Inoue K, Shirano Y, Hayashi H, Shibata D, Sato S, Kato T, Tabata S, Okada K, Wada T (2007) Arabidopsis TRANSPARENT TESTA GLABRA2 is directly regulated by R2R3 MYB transcription factors and is involved in regulation of GLABRA2 transcription in epidermal differentiation. Plant Cell **19:** 2531-2543

Izawa T, Foster R, Chua NH (1993) Plant bZIP protein DNA binding specificity. J Mol Biol **230:** 1131-1144

Jin H, Cominelli E, Bailey P, Parr A, Mehrtens F, Jones J, Tonelli C, Weisshaar B, Martin C (2000) Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in Arabidopsis. EMBO J **19:** 6150-6161

Jones L, Ennos AR, Turner SR (2001) Cloning and characterization of irregular xylem4 (irx4): a severely lignin-deficient mutant of Arabidopsis. Plant J **26:** 205-216

Joshi CP, Mansfield SD (2007) The cellulose paradox--simple molecule, complex biosynthesis. Curr Opin Plant Biol **10:** 220-226

Kanei-Ishii C, Sarai A, Sawazaki T, Nakagoshi H, He DN, Ogata K, Nishimura Y, Ishii S (1990) The tryptophan cluster: a hypothetical structure of the DNA-binding domain of the myb protooncogene product. J Biol Chem **265:** 19990-19995

Kanei-Ishii C, Tanikawa J, Nakai A, Morimoto RI, Ishii S (1997) Activation of heat shock transcription factor 3 by c-Myb in the absence of cellular stress. Science **277:** 246-248

Karpinska B, Karlsson M, Srivastava M, Stenberg A, Schrader J, Sterky F, Bhalerao R, Wingsle G (2004) MYB transcription factors are differentially expressed and regulated during secondary vascular tissue development in hybrid aspen. Plant Mol Biol **56:** 255-270

Kerstetter R, Vollbrecht E, Lowe B, Veit B, Yamaguchi J, Hake S (1994) Sequence analysis and expression patterns divide the maize knotted1-like homeobox genes into two classes. Plant Cell **6:** 1877-1887

Ko JH, Kim WC, Han KH (2009) Ectopic expression of MYB46 identifies transcriptional regulatory genes involved in secondary wall biosynthesis in Arabidopsis. Plant J **60:** 649-665

Koes R, Verweij W, Quattrocchio F (2005) Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci **10:** 236-242

Koornneef M (1981) The complex syndrome of *ttg* mutants. Arabidopsis Inf Serv **18:** 45-51

Kubo M, Udagawa M, Nishikubo N, Horiguchi G, Yamaguchi M, Ito J, Mimura T, Fukuda H, Demura T (2005) Transcription switches for protoxylem and metaxylem vessel formation. Genes Dev **19:** 1855-1860

Kumar R (2006) The roles of BEL1-like proteins in organ morphogenesis in *Arabidopsis thaliana*. PhD thesis. University of British Columbia (UBC), Vancouver

Kunst L, Klenz JE, Martinez-Zapater J, Haughn GW (1989) AP2 Gene Determines the Identity of Perianth Organs in Flowers of *Arabidopsis thaliana*. Plant Cell **1:** 1195-1208

Kuromori T, Hirayama T, Kiyosue Y, Takabe H, Mizukado S, Sakurai T, Akiyama K, Kamiya A, Ito T, Shinozaki K (2004) A collection of 11 800 single-copy Ds transposon insertion lines in Arabidopsis. Plant J **37:** 897-905

Lai LB, Nadeau JA, Lucas J, Lee EK, Nakagawa T, Zhao L, Geisler M, Sack FD (2005) The Arabidopsis R2R3 MYB proteins FOUR LIPS and MYB88 restrict divisions late in the stomatal cell lineage. Plant Cell **17:** 2754-2767

Lea US, Slimestad R, Smedvig P, Lillo C (2007) Nitrogen deficiency enhances expression of specific MYB and bHLH transcription factors and accumulation of end products in the flavonoid pathway. Planta **225:** 1245-1253

Lee D, Meyer K, Chapple C, Douglas CJ (1997) Antisense suppression of 4-coumarate:coenzyme A ligase activity in Arabidopsis leads to altered lignin subunit composition. Plant Cell **9:** 1985-1998

Lee EJ, Matsumura Y, Soga K, Hoson T, Koizumi N (2007) Glycosyl hydrolases of cell wall are induced by sugar starvation in Arabidopsis. Plant Cell Physiol **48:** 405-413

Lee MM, Schiefelbein J (2001) Developmentally distinct MYB genes encode functionally equivalent proteins in Arabidopsis. Development **128:** 1539-1546

Lepiniec L, Debeaujon I, Routaboul JM, Baudry A, Pourcel L, Nesi N, Caboche M (2006) Genetics and biochemistry of seed flavonoids. Annu Rev Plant Biol **57:** 405-430

Lerouxel O, Cavalier DM, Liepman AH, Keegstra K (2006) Biosynthesis of plant cell wall polysaccharides - a complex process. Curr Opin Plant Biol **9:** 621-630

Li E (2009) Identification and characterization of regulatory genes associated with secondary wall formation in Populus and Arabidopsis thaliana. PhD thesis. University of British Columbia,

Li L, Yu X, Thompson A, Guo M, Yoshida S, Asami T, Chory J, Yin Y (2009a) Arabidopsis MYB30 is a direct target of BES1 and cooperates with BES1 to regulate brassinosteroid-induced gene expression. Plant J **58:** 275-286

Li SF, Milliken ON, Pham H, Seyit R, Napoli R, Preston J, Koltunow AM, Parish RW (2009b) The Arabidopsis MYB5 transcription factor regulates mucilage synthesis, seed coat development, and trichome morphogenesis. Plant Cell **21:** 72-89

Liang YK, Dubos C, Dodd IC, Holroyd GH, Hetherington AM, Campbell MM (2005) AtMYB61, an R2R3-MYB transcription factor controlling stomatal aperture in *Arabidopsis thaliana*. Curr Biol **15:** 1201-1206

Lincoln C, Long J, Yamaguchi J, Serikawa K, Hake S (1994) A knotted1-like homeobox gene in Arabidopsis is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. Plant Cell **6:** 1859-1876

Long JA, Moan EI, Medford JI, Barton MK (1996) A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. Nature **379:** 66-69

Lu SX, Knowles SM, Andronis C, Ong MS, Tobin EM (2009) CIRCADIAN CLOCK ASSOCIATED1 and LATE ELONGATED HYPOCOTYL function synergistically in the circadian clock of Arabidopsis. Plant Physiol **150:** 834-843

Malamy JE, Benfey PN (1997) Analysis of SCARECROW expression using a rapid system for assessing transgene expression in Arabidopsis roots. Plant J **12:** 957-963

Marles MA, Ray H, Gruber MY (2003) New perspectives on proanthocyanidin biochemistry and molecular regulation. Phytochemistry **64:** 367-383

Martin C, Glover BJ (2007) Functional aspects of cell patterning in aerial epidermis. Curr Opin Plant Biol **10:** 70-82

Martin C, Paz-Ares J (1997) MYB transcription factors in plants. Trends Genet **13:** 67-73

Matsui K, Tanaka H, Ohme-Takagi M (2004) Suppression of the biosynthesis of proanthocyanidin in Arabidopsis by a chimeric PAP1 repressor. Plant Biotechnol J **2:** 487-493

Matus JT, Aquea F, Arce-Johnson P (2008) Analysis of the grape MYB R2R3 subfamily reveals expanded wine quality-related clades and conserved gene structure organization across Vitis and Arabidopsis genomes. BMC Plant Biol **8:** 83

McCarthy RL, Zhong R, Fowler S, Lyskowski D, Piyasena H, Carleton K, Spicer C, Ye ZH (2010) The poplar MYB transcription factors, PtrMYB3 and PtrMYB20, are involved in the regulation of secondary wall biosynthesis. Plant Cell Physiol **51:** 1084-1090

McCarthy RL, Zhong R, Ye ZH (2009) MYB83 is a direct target of SND1 and acts redundantly with MYB46 in the regulation of secondary cell wall biosynthesis in Arabidopsis. Plant Cell Physiol **50:** 1950-1964

McGinnis W, Krumlauf R (1992) Homeobox genes and axial patterning. Cell **68:** 283-302

Mehrtens F, Kranz H, Bednarek P, Weisshaar B (2005) The Arabidopsis transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid biosynthesis. Plant Physiol **138:** 1083-1096

Meisel L, Lam E (1996) The conserved ELK-homeodomain of KNOTTED-1 contains two regions that signal nuclear localization. Plant Mol Biol **30:** 1-14

Meissner RC, Jin H, Cominelli E, Denekamp M, Fuertes A, Greco R, Kranz HD, Penfield S, Petroni K, Urzainqui A, Martin C, Paz-Ares J, Smeekens S, Tonelli C, Weisshaar B,

Baumann E, Klimyuk V, Marillonnet S, Patel K, Speulman E, Tissier AF, Bouchez D, Jones JJ, Pereira A, Wisman E (1999) Function search in a large transcription factor gene family in Arabidopsis: assessing the potential of reverse genetics to identify insertional mutations in R2R3 MYB genes. Plant Cell **11:** 1827-1840

Menkens AE, Schindler U, Cashmore AR (1995) The G-box: a ubiquitous regulatory DNA element in plants bound by the GBF family of bZIP proteins. Trends Biochem Sci **20:** 506-510

Meshi T, Iwabuchi M (1995) Plant transcription factors. Plant Cell Physiol **36:** 1405-1420

Meyer K, Shirley AM, Cusumano JC, Bell-Lelong DA, Chapple C (1998) Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenase in Arabidopsis. Proc Natl Acad Sci U S A **95:** 6619-6623

Mir Derikvand M, Sierra JB, Ruel K, Pollet B, Do CT, Thevenin J, Buffard D, Jouanin L, Lapierre C (2008) Redirection of the phenylpropanoid pathway to feruloyl malate in Arabidopsis mutants deficient for cinnamoyl-CoA reductase 1. Planta **227:** 943-956

Misson J, Raghothama KG, Jain A, Jouhet J, Block MA, Bligny R, Ortet P, Creff A, Somerville S, Rolland N, Doumas P, Nacry P, Herrerra-Estrella L, Nussaume L, Thibaud MC (2005) A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. Proc Natl Acad Sci U S A **102:** 11934-11939

Mitsuda N, Iwase A, Yamamoto H, Yoshida M, Seki M, Shinozaki K, Ohme-Takagi M (2007) NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of Arabidopsis. Plant Cell **19:** 270-280

Mitsuda N, Ohme-Takagi M (2009) Functional analysis of transcription factors in Arabidopsis. Plant Cell Physiol **50:** 1232-1248

Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M (2005) The NAC transcription factors NST1 and NST2 of Arabidopsis regulate secondary wall thickenings and are required for anther dehiscence. Plant Cell **17:** 2993-3006

Morcuende R, Bari R, Gibon Y, Zheng W, Pant BD, Blasing O, Usadel B, Czechowski T, Udvardi MK, Stitt M, Scheible WR (2007) Genome-wide reprogramming of metabolism and regulatory networks of Arabidopsis in response to phosphorus. Plant Cell Environ **30:** 85-112

Morita Y, Saitoh M, Hoshino A, Nitasaka E, Iida S (2006) Isolation of cDNAs for R2R3-MYB, bHLH and WDR transcriptional regulators and identification of c and ca mutations conferring white flowers in the Japanese morning glory. Plant Cell Physiol **47:** 457-470

Mukherjee K, Brocchieri L, Burglin TR (2009) A comprehensive classification and evolutionary analysis of plant homeobox genes. Mol Biol Evol **26:** 2775-2794

Mukherjee K, Burglin TR (2007) Comprehensive analysis of animal TALE homeobox genes: new conserved motifs and cases of accelerated evolution. J Mol Evol **65:** 137-153

Muller J, Wang Y, Franzen R, Santi L, Salamini F, Rohde W (2001) In vitro interactions between barley TALE homeodomain proteins suggest a role for protein-protein associations in the regulation of Knox gene function. Plant J **27:** 13-23

Muller R, Morant M, Jarmer H, Nilsson L, Nielsen TH (2007) Genome-wide analysis of the Arabidopsis leaf transcriptome reveals interaction of phosphate and sugar metabolism. Plant Physiol **143:** 156-171

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473-497

Nagasaki H, Sakamoto T, Sato Y, Matsuoka M (2001) Functional analysis of the conserved domains of a rice KNOX homeodomain protein, OSH15. Plant Cell **13:** 2085-2098

Neff MM, Chory J (1998) Genetic interactions between phytochrome A, phytochrome B, and cryptochrome 1 during Arabidopsis development. Plant Physiol **118:** 27-35

Nesi N, Debeaujon I, Jond C, Pelletier G, Caboche M, Lepiniec L (2000) The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of DFR and BAN genes in Arabidopsis siliques. Plant Cell **12:** 1863-1878

Nesi N, Jond C, Debeaujon I, Caboche M, Lepiniec L (2001) The Arabidopsis TT2 gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. Plant Cell **13:** 2099-2114

Newman LJ, Perazza DE, Juda L, Campbell MM (2004) Involvement of the R2R3-MYB, AtMYB61, in the ectopic lignification and dark-photomorphogenic components of the det3 mutant phenotype. Plant J **37:** 239-250

Niggeweg R, Thurow C, Kegler C, Gatz C (2000) Tobacco transcription factor TGA2.2 is the main component of as-1-binding factor ASF-1 and is involved in salicylic acid- and auxin-inducible expression of as-1-containing target promoters. J Biol Chem **275:** 19897-19905

Noda K, Glover BJ, Linstead P, Martin C (1994) Flower colour intensity depends on specialized cell shape controlled by a Myb-related transcription factor. Nature **369:** 661-664

O'Neill MA, York WS (2003) The composition and structure of plant primary cell walls.

Oda Y, Mimura T, Hasezawa S (2005) Regulation of secondary cell wall development by cortical microtubules during tracheary element differentiation in Arabidopsis cell suspensions. Plant Physiol **137:** 1027-1036

Ogata K, Morikawa S, Nakamura H, Hojo H, Yoshimura S, Zhang R, Aimoto S, Ametani Y, Hirata Z, Sarai A (1995) Comparison of the free and DNA-complexed forms of the DNA-binding domain from c-Myb. Nat Struct Biol **2:** 309-320

Ohto MA, Floyd SK, Fischer RL, Goldberg RB, Harada JJ (2009) Effects of APETALA2 on embryo, endosperm, and seed coat development determine seed size in Arabidopsis. Sex Plant Reprod **22:** 277-289

Oka T, Nemoto T, Jigami Y (2007) Functional analysis of *Arabidopsis thaliana* RHM2/MUM4, a multidomain protein involved in UDP-D-glucose to UDP-L-rhamnose conversion. J Biol Chem **282:** 5389-5403

Oppenheimer DG, Herman PL, Sivakumaran S, Esch J, Marks MD (1991) A myb gene required for leaf trichome differentiation in Arabidopsis is expressed in stipules. Cell **67:** 483-493

Pabo CO, Sauer RT (1992) Transcription factors: structural families and principles of DNA recognition. Annu Rev Biochem **61:** 1053-1095

Palapol Y, Ketsa S, Lin-Wang K, Ferguson IB, Allan AC (2009) A MYB transcription factor regulates anthocyanin biosynthesis in mangosteen (*Garcinia mangostana L.*) fruit during ripening. Planta **229:** 1323-1334

Patzlaff A, McInnis S, Courtenay A, Surman C, Newman LJ, Smith C, Bevan MW, Mansfield S, Whetten RW, Sederoff RR, Campbell MM (2003a) Characterisation of a pine MYB that regulates lignification. Plant J **36:** 743-754

Patzlaff A, Newman LJ, Dubos C, Whetten RW, Smith C, McInnis S, Bevan MW, Sederoff RR, Campbell MM (2003b) Characterisation of Pt MYB1, an R2R3-MYB from pine xylem. Plant Mol Biol **53:** 597-608

Pauly M, Keegstra K (2008) Cell-wall carbohydrates and their modification as a resource for biofuels. Plant J **54:** 559-568

Payne CT, Zhang F, Lloyd AM (2000) GL3 encodes a bHLH protein that regulates trichome development in Arabidopsis through interaction with GL1 and TTG1. Genetics **156:** 1349-1362

Pellegrini L, Tan S, Richmond TJ (1995) Structure of serum response factor core bound to DNA. Nature **376:** 490-498

Pena MJ, Zhong R, Zhou GK, Richardson EA, O'Neill MA, Darvill AG, York WS, Ye ZH (2007) Arabidopsis *irregular xylem8* and *irregular xylem9*: implications for the complexity of glucuronoxylan biosynthesis. Plant Cell **19:** 549-563

Penfield S, Meissner RC, Shoue DA, Carpita NC, Bevan MW (2001) MYB61 is required for mucilage deposition and extrusion in the Arabidopsis seed coat. Plant Cell **13:** 2777-2791

Persson S, Caffall KH, Freshour G, Hilley MT, Bauer S, Poindexter P, Hahn MG, Mohnen D, Somerville C (2007) The Arabidopsis irregular xylem8 mutant is deficient in glucuronoxylan and homogalacturonan, which are essential for secondary cell wall integrity. Plant Cell **19:** 237-255

Persson S, Wei H, Milne J, Page GP, Somerville CR (2005) Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets. Proc Natl Acad Sci U S A **102:** 8633-8638

Pontier D, Miao ZH, Lam E (2001) Trans-dominant suppression of plant TGA factors reveals their negative and positive roles in plant defense responses. Plant J **27:** 529-538

Pourtau N, Jennings R, Pelzer E, Pallas J, Wingler A (2006) Effect of sugar-induced senescence on gene expression and implications for the regulation of senescence in Arabidopsis. Planta **224:** 556-568

Preston J, Wheeler J, Heazlewood J, Li SF, Parish RW (2004) AtMYB32 is required for normal pollen development in *Arabidopsis thaliana*. Plant J **40:** 979-995

Qu LJ, Zhu YX (2006) Transcription factor families in Arabidopsis: major progress and outstanding issues for future research. Curr Opin Plant Biol **9:** 544-549

Quattrocchio F, Verweij W, Kroon A, Spelt C, Mol J, Koes R (2006) PH4 of Petunia is an R2R3 MYB protein that activates vacuolar acidification through interactions with basic-helix-loop-helix transcription factors of the anthocyanin pathway. Plant Cell **18:** 1274-1291

Raes J, Rohde A, Christensen JH, Van de Peer Y, Boerjan W (2003) Genome-wide characterization of the lignification toolbox in Arabidopsis. Plant Physiol **133:** 1051-1071

Ramsay NA, Glover BJ (2005) MYB-bHLH-WD40 protein complex and the evolution of cellular diversity. Trends Plant Sci **10:** 63-70

Raven PH, Evert RF, Eichorn SE (1999) The Biology of Plants, Ed 6. W.H. Freeman Worth Publishers, New York

Reiser L, Sanchez-Baracaldo P, Hake S (2000) Knots in the family tree: evolutionary relationships and functions of knox homeobox genes. Plant Mol Biol **42:** 151-166

Riechmann JL, Heard J, Martin G, Reuber L, Jiang C, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR, Creelman R, Pilgrim M, Broun P, Zhang JZ, Ghandehari D, Sherman BK, Yu G (2000) Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. Science **290:** 2105-2110

Riechmann JL, Meyerowitz EM (1998) The AP2/EREBP family of plant transcription factors. Biol Chem **379:** 633-646

Riechmann JL, Meyerowitz EM (1997) MADS domain proteins in plant development. Biol Chem **378:** 1079-1101

Roberts AW, Frost AO, Roberts EM, Haigler CH (2004) Roles of microtubules and cellulose microfibril assembly in the localization of secondary-cell-wall deposition in developing tracheary elements. Protoplasma **224:** 217-229

Robinson AR, Mansfield SD (2009) Rapid analysis of poplar lignin monomer composition by a streamlined thioacidolysis procedure and near-infrared reflectance-based prediction modeling. Plant J **58:** 706-714

Rogers LA, Dubos C, Cullis IF, Surman C, Poole M, Willment J, Mansfield SD, Campbell MM (2005) Light, the circadian clock, and sugar perception in the control of lignin biosynthesis. J Exp Bot **56:** 1651-1663

Rosinski JA, Atchley WR (1999) Molecular evolution of helix-turn-helix proteins. J Mol Evol **49:** 301-309

Rosinski JA, Atchley WR (1998) Molecular evolution of the Myb family of transcription factors: evidence for polyphyletic origin. J Mol Evol **46:** 74-83

Rowan DD, Cao M, Lin-Wang K, Cooney JM, Jensen DJ, Austin PT, Hunt MB, Norling C, Hellens RP, Schaffer RJ, Allan AC (2009) Environmental regulation of leaf colour in red 35S:PAP1 *Arabidopsis thaliana*. New Phytol **182:** 102-115

Rubio V, Linhares F, Solano R, Martin AC, Iglesias J, Leyva A, Paz-Ares J (2001) A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. Genes Dev **15:** 2122-2133

Rubio V, Shen Y, Saijo Y, Liu Y, Gusmaroli G, Dinesh-Kumar SP, Deng XW (2005) An alternative tandem affinity purification strategy applied to Arabidopsis protein complex isolation. Plant J **41:** 767-778

Ruel K, Berrio-Sierra J, Derikvand MM, Pollet B, Thevenin J, Lapierre C, Jouanin L, Joseleau JP (2009) Impact of CCR1 silencing on the assembly of lignified secondary walls in *Arabidopsis thaliana*. New Phytol **184:** 99-113

Sakakibara K, Nishiyama T, Deguchi H, Hasebe M (2008) Class 1 KNOX genes are not involved in shoot development in the moss *Physcomitrella patens* but do function in sporophyte development. Evol Dev **10:** 555-566

Schellmann S, Hulskamp M (2005) Epidermal differentiation: trichomes in Arabidopsis as a model system. Int J Dev Biol **49:** 579-584

Schiefelbein JW (2000) Constructing a plant cell. The genetic control of root hair development. Plant Physiol **124:** 1525-1531

Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Scholkopf B, Weigel D, Lohmann JU (2005) A gene expression map of *Arabidopsis thaliana* development. Nat Genet **37:** 501-506

Schutze K, Harter K, Chaban C (2009) Bimolecular fluorescence complementation (BiFC) to study protein-protein interactions in living plant cells. Methods Mol Biol **479:** 189-202

Schwab R, Ossowski S, Warthmann N, Weigel D (2010) Directed gene silencing with artificial microRNAs. Methods Mol Biol **592:** 71-88

Schwechheimer C, Zourelidou M, Bevan MW (1998) Plant Transcription Factor Studies. Annu Rev Plant Physiol Plant Mol Biol **49:** 127-150

Semiarti E, Ueno Y, Tsukaya H, Iwakawa H, Machida C, Machida Y (2001) The ASYMMETRIC LEAVES2 gene of *Arabidopsis thaliana* regulates formation of a symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves. Development **128:** 1771-1783

Serna L (2005) Epidermal cell patterning and differentiation throughout the apical-basal axis of the seedling. J Exp Bot **56:** 1983-1989

Serna L, Martin C (2006) Trichomes: different regulatory networks lead to convergent structures. Trends Plant Sci **11:** 274-280

Shin R, Burch AY, Huppert KA, Tiwari SB, Murphy AS, Guilfoyle TJ, Schachtman DP (2007) The Arabidopsis transcription factor MYB77 modulates auxin signal transduction. Plant Cell **19:** 2440-2453

Sibout R, Eudes A, Mouille G, Pollet B, Lapierre C, Jouanin L, Seguin A (2005) CINNAMYL ALCOHOL DEHYDROGENASE-C and -D are the primary genes involved in lignin biosynthesis in the floral stem of Arabidopsis. Plant Cell **17:** 2059-2076

Singh KB (1998) Transcriptional regulation in plants: the importance of combinatorial control. Plant Physiol **118:** 1111-1120

Singh S, Lewis NG, Towers GH (1998) Nitrogen recycling during phenylpropanoid metabolism in sweet potato tubers. J Plant Physiol **153:** 316-323

Smith AM, Stitt M (2007) Coordination of carbon supply and plant growth. Plant Cell Environ **30:** 1126-1149

Smith HM, Boschke I, Hake S (2002) Selective interaction of plant homeodomain proteins mediates high DNA-binding affinity. Proc Natl Acad Sci U S A **99:** 9579-9584

Somerville C (2006) Cellulose synthesis in higher plants. Annu Rev Cell Dev Biol **22:** 53-78

Somerville C, Bauer S, Brininstool G, Facette M, Hamann T, Milne J, Osborne E, Paredez A, Persson S, Raab T, Vorwerk S, Youngs H (2004) Toward a systems approach to understanding plant cell walls. Science **306:** 2206-2211

Sterling C (1970) Crystal-structure of ruthenium red and stereochemistry of its pectic stain. Amer J Bot **57:** 175

Stober-Grasser U, Brydolf B, Bin X, Grasser F, Firtel RA, Lipsick JS (1992) The Myb DNA-binding domain is highly conserved in *Dictyostelium discoideum*. Oncogene **7:** 589-596

Storey J (2002) A direct approach to false discovery rates. J R Stat Soc Ser B Stat Methodol **64:** 479-498

Stork J, Harris D, Griffiths J, Williams B, Beisson F, Li-Beisson Y, Mendu V, Haughn G, Debolt S (2010) CELLULOSE SYNTHASE9 serves a nonredundant role in secondary cell wall synthesis in Arabidopsis epidermal testa cells. Plant Physiol **153:** 580-589

Stracke R, Ishihara H, Huep G, Barsch A, Mehrtens F, Niehaus K, Weisshaar B (2007a) Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. Plant J

Stracke R, Werber M, Weisshaar B (2001) The R2R3-MYB gene family in *Arabidopsis thaliana*. Curr Opin Plant Biol **4:** 447-456

Sugimoto K, Himmelspach R, Williamson RE, Wasteneys GO (2003) Mutation or drug-dependent microtubule disruption causes radial swelling without altering parallel cellulose microfibril deposition in Arabidopsis root cells. Plant Cell **15:** 1414-1429

Sugimoto K, Takeda S, Hirochika H (2000) MYB-related transcription factor NtMYB2 induced by wounding and elicitors is a regulator of the tobacco retrotransposon Tto1 and defense-related genes. Plant Cell **12:** 2511-2528

Takatsuji H (1999) Zinc-finger proteins: the classical zinc finger emerges in contemporary plant science. Plant Mol Biol **39:** 1073-1078

Tamagnone L, Merida A, Parr A, Mackay S, Culianez-Macia FA, Roberts K, Martin C (1998) The AmMYB308 and AmMYB330 transcription factors from antirrhinum regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. Plant Cell **10:** 135-154

Tan K, Shlomi T, Feizi H, Ideker T, Sharan R (2007) Transcriptional regulation of protein complexes within and across species. Proc Natl Acad Sci U S A **104:** 1283-1288

Taylor NG, Gardiner JC, Whiteman R, Turner SR (2004) Cellulose synthesis in the *Arabidopsis* secondary cell wall Cellulose **11:** 329-338

Taylor NG, Howells RM, Huttly AK, Vickers K, Turner SR (2003) Interactions among three distinct CesA proteins essential for cellulose synthesis. Proc Natl Acad Sci U S A **100:** 1450-1455

Teng S, Keurentjes J, Bentsink L, Koornneef M, Smeekens S (2005) Sucrose-specific induction of anthocyanin biosynthesis in Arabidopsis requires the MYB75/PAP1 gene. Plant Physiol **139:** 1840-1852

Theissen G, Kim JT, Saedler H (1996) Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes. J Mol Evol **43:** 484-516

Timmermans MC, Hudson A, Becraft PW, Nelson T (1999) ROUGH SHEATH2: a Myb protein that represses knox homeobox genes in maize lateral organ primordia. Science **284:** 151-153

Tiwari S, Wang S, Hagen G, Guilfoyle TJ (2006) Transfection assays with protoplasts containing integrated reporter genes. Methods Mol Biol **323:** 237-244

Tohge T, Matsui K, Ohme-Takagi M, Yamazaki M, Saito K (2005a) Enhanced radical scavenging activity of genetically modified Arabidopsis seeds. Biotechnol Lett **27:** 297-303

Tohge T, Nishiyama Y, Hirai MY, Yano M, Nakajima J, Awazuhara M, Inoue E, Takahashi H, Goodenowe DB, Kitayama M, Noji M, Yamazaki M, Saito K (2005b) Functional genomics by integrated analysis of metabolome and transcriptome of Arabidopsis plants over-expressing an MYB transcription factor. Plant J **42:** 218-235

Turner SR, Somerville CR (1997) Collapsed xylem phenotype of Arabidopsis identifies mutants deficient in cellulose deposition in the secondary cell wall. Plant Cell **9:** 689-701

Uhrig JF (2006) Protein interaction networks in plants. Planta **224:** 771-781

Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An Arabidopsis myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell **5:** 1529-1539

Usadel B, Kuschinsky AM, Rosso MG, Eckermann N, Pauly M (2004) RHM2 is involved in mucilage pectin synthesis and is required for the development of the seed coat in Arabidopsis. Plant Physiol **134:** 286-295

Vanderauwera S, Zimmermann P, Rombauts S, Vandenabeele S, Langebartels C, Gruissem W, Inze D, Van Breusegem F (2005) Genome-wide analysis of hydrogen peroxide-regulated gene expression in Arabidopsis reveals a high light-induced transcriptional cluster involved in anthocyanin biosynthesis. Plant Physiol **139:** 806-821

Viola IL, Gonzalez DH (2006) Interaction of the BELL-like protein ATH1 with DNA: role of homeodomain residue 54 in specifying the different binding properties of BELL and KNOX proteins. Biol Chem **387:** 31-40

Walhout AJ, Sordella R, Lu X, Hartley JL, Temple GF, Brasch MA, Thierry-Mieg N, Vidal M (2000) Protein interaction mapping in *C. elegans* using proteins involved in vulval development. Science **287:** 116-122

Walter M, Chaban C, Schutze K, Batistic O, Weckermann K, Nake C, Blazevic D, Grefen C, Schumacher K, Oecking C, Harter K, Kudla J (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. Plant J **40:** 428-438

Wang S, Chang Y, Guo J, Chen JG (2007) Arabidopsis Ovate Family Protein 1 is a transcriptional repressor that suppresses cell elongation. Plant J **50:** 858-872

Wasteneys GO (2004) Progress in understanding the role of microtubules in plant cells. Curr Opin Plant Biol **7:** 651-660

Weinthal D, Tzfira T (2009) Imaging protein-protein interactions in plant cells by bimolecular fluorescence complementation assay. Trends Plant Sci **14:** 59-63

Western TJ (2006) Changing spaces: The Arabidopsis mucilage secretory cells as a novel system to dissect cell wall production in differentiating cells. Can J Bot **84:** 622-630

Western TL, Burn J, Tan WL, Skinner DJ, Martin-McCaffrey L, Moffatt BA, Haughn GW (2001) Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in Arabidopsis. Plant Physiol **127:** 998-1011

Western TL, Skinner DJ, Haughn GW (2000) Differentiation of mucilage secretory cells of the Arabidopsis seed coat. Plant Physiol **122:** 345-356

Western TL, Young DS, Dean GH, Tan WL, Samuels AL, Haughn GW (2004) MUCILAGE-MODIFIED4 encodes a putative pectin biosynthetic enzyme developmentally regulated by APETALA2, TRANSPARENT TESTA GLABRA1, and GLABRA2 in the Arabidopsis seed coat. Plant Physiol **134:** 296-306

Wightman R, Turner SR (2008) The roles of the cytoskeleton during cellulose deposition at the secondary cell wall. Plant J **54:** 794-805

Wilkins O, Nahal H, Foong J, Provart NJ, Campbell MM (2009) Expansion and diversification of the Populus R2R3-MYB family of transcription factors. Plant Physiol **149:** 981-993

Wolberger C (1999) Multiprotein-DNA complexes in transcriptional regulation. Annu Rev Biophys Biomol Struct **28:** 29-56

Yamaguchi M, Goue N, Igarashi H, Ohtani M, Nakano Y, Mortimer JC, Nishikubo N, Kubo M, Katayama Y, Kakegawa K, Dupree P, Demura T (2010) VASCULAR-RELATED NAC-DOMAIN6 and VASCULAR-RELATED NAC-DOMAIN7 effectively induce transdifferentiation into xylem vessel elements under control of an induction system. Plant Physiol **153:** 906-914

Yamaguchi M, Kubo M, Fukuda H, Demura T (2008) Vascular-related NAC-DOMAIN7 is involved in the differentiation of all types of xylem vessels in Arabidopsis roots and shoots. Plant J **55:** 652-664

Yanagisawa S, Akiyama A, Kisaka H, Uchimiya H, Miwa T (2004) Metabolic engineering with Dof1 transcription factor in plants: Improved nitrogen assimilation and growth under low-nitrogen conditions. Proc Natl Acad Sci U S A **101:** 7833-7838

Yanagisawa S, Schmidt RJ (1999) Diversity and similarity among recognition sequences of Dof transcription factors. Plant J **17:** 209-214

Yang C, Xu Z, Song J, Conner K, Vizcay Barrena G, Wilson ZA (2007) Arabidopsis MYB26/MALE STERILE35 regulates secondary thickening in the endothecium and is essential for anther dehiscence. Plant Cell **19:** 534-548

Yang L, Liu Z, Lu F, Dong A, Huang H (2006) SERRATE is a novel nuclear regulator in primary microRNA processing in Arabidopsis. Plant J **47:** 841-850

Yang SW, Jang IC, Henriques R, Chua NH (2009) FAR-RED ELONGATED HYPOCOTYL1 and FHY1-LIKE associate with the Arabidopsis transcription factors LAF1 and HFR1 to transmit phytochrome A signals for inhibition of hypocotyl elongation. Plant Cell **21:** 1341-1359

Yang Y, Klessig DF (1996) Isolation and characterization of a tobacco mosaic virus-inducible myb oncogene homolog from tobacco. Proc Natl Acad Sci U S A **93:** 14972-14977

Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, Speed TP (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res **30:** e15

Yanhui C, Xiaoyuan Y, Kun H, Meihua L, Jigang L, Zhaofeng G, Zhiqiang L, Yunfei Z, Xiaoxiao W, Xiaoming Q, Yunping S, Li Z, Xiaohui D, Jingchu L, Xing-Wang D, Zhangliang C, Hongya G, Li-Jia Q (2006) The MYB transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family. Plant Mol Biol **60:** 107-124

Ye ZH, Freshour G, Hahn MG, Burk DH, Zhong R (2002) Vascular development in Arabidopsis. Int Rev Cytol **220:** 225-256

Yokoyama R, Nishitani K (2006) Identification and characterization of *Arabidopsis thaliana* genes involved in xylem secondary cell walls. J Plant Res **119:** 189-194

Zhang F, Gonzalez A, Zhao M, Payne CT, Lloyd A (2003) A network of redundant bHLH proteins functions in all TTG1-dependent pathways of Arabidopsis. Development **130:** 4859-4869

Zhang H, Liang W, Yang X, Luo X, Jiang N, Ma H, Zhang D (2010) Carbon starved anther encodes a MYB domain protein that regulates sugar partitioning required for rice pollen development. Plant Cell **22:** 672-689

Zhang Y, Brown G, Whetten R, Loopstra CA, Neale D, Kieliszewski MJ, Sederoff RR (2003) An arabinogalactan protein associated with secondary cell wall formation in differentiating xylem of loblolly pine. Plant Mol Biol **52:** 91-102

Zhang ZB, Zhu J, Gao JF, Wang C, Li H, Li H, Zhang HQ, Zhang S, Wang DM, Wang QX, Huang H, Xia HJ, Yang ZN (2007) Transcription factor AtMYB103 is required for anther development by regulating tapetum development, callose dissolution and exine formation in Arabidopsis. Plant J **52:** 528-538

Zhao C, Avci U, Grant EH, Haigler CH, Beers EP (2008) XND1, a member of the NAC domain family in *Arabidopsis thaliana*, negatively regulates lignocellulose synthesis and programmed cell death in xylem. Plant J **53:** 425-436

Zhao C, Craig JC, Petzold HE, Dickerman AW, Beers EP (2005) The xylem and phloem transcriptomes from secondary tissues of the Arabidopsis root-hypocotyl. Plant Physiol **138:** 803-818

Zhao M, Morohashi K, Hatlestad G, Grotewold E, Lloyd A (2008) The TTG1-bHLH-MYB complex controls trichome cell fate and patterning through direct targeting of regulatory loci. Development **135:** 1991-1999

Zheng ZL (2009) Carbon and nitrogen nutrient balance signaling in plants. Plant Signal Behav **4:** 584-591

Zhong R, Demura T, Ye ZH (2006) SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of Arabidopsis. Plant Cell **18:** 3158-3170

Zhong R, III WH, Negrel J, Ye ZH (1998) Dual methylation pathways in lignin biosynthesis. Plant Cell **10:** 2033-2046

Zhong R, Lee C, Zhou J, McCarthy RL, Ye ZH (2008) A battery of transcription factors involved in the regulation of secondary cell wall biosynthesis in Arabidopsis. Plant Cell **20:** 2763-2782

Zhong R, Pena MJ, Zhou GK, Nairn CJ, Wood-Jones A, Richardson EA, Morrison WH,3rd, Darvill AG, York WS, Ye ZH (2005) Arabidopsis *fragile fiber8*, which encodes a putative glucuronyltransferase, is essential for normal secondary wall synthesis. Plant Cell **17:** 3390-3408

Zhong R, Richardson EA, Ye ZH (2007a) The MYB46 transcription factor is a direct target of SND1 and regulates secondary wall biosynthesis in Arabidopsis. Plant Cell **19:** 2776-2792

Zhong R, Richardson EA, Ye ZH (2007b) Two NAC domain transcription factors, SND1 and NST1, function redundantly in regulation of secondary wall synthesis in fibers of Arabidopsis. Planta **225:** 1603-1611

Zhong R, Ye ZH (2009) Transcriptional regulation of lignin biosynthesis. Plant Signal Behav **4:** 1028-1034

Zhong R, Ye ZH (2007) Regulation of cell wall biosynthesis. Curr Opin Plant Biol **10:** 564-572

Zhou J, Lee C, Zhong R, Ye ZH (2009) MYB58 and MYB63 are transcriptional activators of the lignin biosynthetic pathway during secondary cell wall formation in Arabidopsis. Plant Cell **21:** 248-266

Zimmermann IM, Heim MA, Weisshaar B, Uhrig JF (2004) Comprehensive identification of *Arabidopsis thaliana* MYB transcription factors interacting with R/B-like BHLH proteins. Plant J **40:** 22-34