Investigation of a KNAT7-B LH-OFP transcription factor complex involved in regulation of secondary cell wall biosynthesis in Arabidopsis thaliana

by

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ABSTRACT

The plant secondary cell wall is a composite network of complex polymers (cellulose, lignin, and hemicellulose) that provides protective and structural properties to the cell wall. Based on previous research, the *Arabidopsis* KNOX gene KNAT7 has been shown to act as a transcription factor that regulates secondary wall formation in *Arabidopsis* inflorescence stems in coordination with Ovate Family Proteins (OFPs). Co-expression and yeast two-hybrid analyses suggest that BEL1-LIKE HOMEODOMAIN (BLH) transcription factors could be part of a KNOX-BLH-OVATE transcription factor complex regulating aspects of secondary cell wall formation, together with KNAT7 and OFP1/4. I investigated the interactions of BLH partners with KNAT7 and OFP proteins through yeast two-hybrid and *in planta* bimolecular fluorescence complementation analyses, and have identified a BLH protein BLH6 (At4g34610), from among six candidate BLH proteins as a BLH interacting partner of KNAT7. In addition, I demonstrated that OFP4 interacts with homeodomain of KNAT7 and BLH6 interacts with the KNAT7 MEINOX domain by yeast two-hybrid analyses. Furthermore, I investigated the function of BLH6 and an additional BLH protein, BLH5 (At2g27220), by characterizing the phenotypic effects of *blh* loss of function and *BLH* overexpression on stem anatomy. Phenotype analysis showed that *blh5* knockout mutant and *BLH5* overexpression mutant are indistinguishable from wild type. *blh6* knock out mutant displayed slightly thicker cell walls in interfascicular fibers. In addition, I employed protoplast transfection assay to demonstrate that BLH6 is a transcriptional repressor. This study provides new information regarding the existence of a BLH6-KNAT7-OFP complex and insights into the biological function of BLH6.
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### ABBREVIATIONS

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3AT</td>
<td>3-amino-1, 2, 4-triazole</td>
</tr>
<tr>
<td>35S</td>
<td>Cauliflower mosaic virus 35S promoter</td>
</tr>
<tr>
<td>4CL1</td>
<td><em>Petroselinum crispum</em> 4-Coumarate:CoA ligase1</td>
</tr>
<tr>
<td>ABRC</td>
<td>Arabidopsis Biological Resource Center</td>
</tr>
<tr>
<td>BLH</td>
<td>BEL1-like homeodomain (protein/gene)</td>
</tr>
<tr>
<td>BEL1</td>
<td>BELL1</td>
</tr>
<tr>
<td>BAR</td>
<td>Bio-Array Resource for Arabidopsis Functional Genomics</td>
</tr>
<tr>
<td>BiFC</td>
<td>Bimolecular fluorescence complementation</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>CEYFP/NEYFP</td>
<td>C terminus / N terminus of enhanced yellow fluorescent protein</td>
</tr>
<tr>
<td>CESA</td>
<td>Cellulose synthase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA reverse transcribed from messenger RNA (mRNA)</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>EV</td>
<td>Empty vector</td>
</tr>
<tr>
<td>FRA8</td>
<td>Arabidopsis thaliana fragile fiber 8</td>
</tr>
<tr>
<td>GD</td>
<td>Gal4 DNA binding domain</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>hr</td>
<td>Hours</td>
</tr>
<tr>
<td>IFF</td>
<td>Interfascicular fiber</td>
</tr>
<tr>
<td>irx</td>
<td>Irregular xylem</td>
</tr>
<tr>
<td>KNOX</td>
<td>Knotted - like homeobox</td>
</tr>
<tr>
<td>KNAT</td>
<td>Knotted - like Arabidopsis thaliana (protein/gene)</td>
</tr>
<tr>
<td>LB</td>
<td>Luria - Bertani bacterial growth medium</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MUG</td>
<td>4-methylumbelliferyl-β-D-glucuronide</td>
</tr>
<tr>
<td>OFP</td>
<td>Ovate family protein</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>TE</td>
<td>Tracheary element</td>
</tr>
<tr>
<td>TALE</td>
<td>Three amino acid loop extension</td>
</tr>
<tr>
<td>T-DNA</td>
<td>Transfer DNA</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>Y2H</td>
<td>Yeast two hybrid</td>
</tr>
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</table>
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Chapter 1. Introduction and literature review

1.1 Xylem development and tracheary element differentiation

Xylem is composed of several cell types, such as tracheary elements (TEs), fibers and parenchyma. TEs in gymnosperms include tracheids, while TEs in angiosperms include both vessels and tracheids. Xylem is produced during primary and secondary growth. Primary xylem is produced via the procambium from the shoot apical meristem, and secondary xylem (wood) is formed from vascular cambium which originates from procambium (Esau, 1965). Xylem cells such as vessels and tracheids that are dead at maturity and have lignified secondary cell walls undergo a common set of developmental steps during xylem development (xylogenesis) (Samuels et al., 2006). Zinnia elegans TEs grown in cell culture have been used as a model system to study xylem differentiation steps such as secondary wall deposition, lignification and programmed cell death (Stacey et al., 1995; Fukuda, 1996). Figure 1.1 summarizes the process of primary xylem development in the model system Zinnia elegans (Samuels et al., 2006). The inflorescence stem of Arabidopsis also serves as a model system to study secondary cell wall development in xylem and interfascicular fiber cells as it displays a gradient of fiber development at increasing distances from shoot apical meristem, and the xylem and interfascicular fiber anatomy and morphology are easily visualized in cross sections (Ehlting et al., 2005). Populus (poplar) has also been used as a model system for wood (secondary xylem) formation in recent years as its genome has been sequenced, genomic and molecular tools have been developed, and it can be transformed (Mellerowicz et al., 2001; Jansson and Douglas, 2007).

Primary and secondary xylem differentiation begins with cell expansion, followed by secondary cell wall synthesis and deposition, lignification, and programmed cell death (PCD) (Turner et al., 2007). During primary xylem development, procambium cells derived from the shoot apical meristem start to expand longitudinally, giving rise to TEs (Figure 1.1). During secondary xylem development, after mitosis of cambial cells, the derivatives that will become xylem first undergo
a period of cellular expansion. Angiosperm vessels and gymnosperm tracheids, which are water conducting cells, undergo radial expansion, while angiosperm supportive fibers undergo intrusive elongation (Mellerowicz et al., 2001). At the end of cell expansion phase, TEs and fiber cells begin to produce a three-layered secondary cell wall (S1, S2 and S3 layers), which is made of cellulose and hemicelluloses. At the later stages of cellulose and hemicelluloses biosynthesis, lignification of cell corners and the middle lamella of TE and fibers begins (Donaldson, 2001). The final stage of xylem cell development is PCD, a process that removes the cell contents and leaves empty cells, which are capable of supporting water transport (Figure 1.2) (Fukuda, 1996; Roberts and McCann, 2000).

**Figure 1.1 Primary xylem development in the model system of Zinnia elegans.**

Xylogenesis processes are very similar in primary xylem lignified cells and secondary xylem, although secondary xylem is produced by vascular cambium. Figure from Samuels et al. (2006).

Primary xylem development - *Zinnia* tracheary element in vitro model
1.2 Secondary cell walls

The evolution of lignified secondary cell walls was a crucial adaptive event in land plant evolution, as it provides structural stiffness and strength to plant cells, allowing for their vertical growth, protecting against pathogen attack and facilitating the transport of water and nutrients through lignified tracheids and vessel elements in the xylem (Roberts and McCann, 2000). Secondary walls also have a major impact on human life, as they are major constituents of wood.
and forage crop biomass. In the future, secondary walls may help to reduce our dependence on petroleum, as they account for the bulk of renewable biomass that can be used for bioenergy (Pauly and Keegstra, 2008).

The plant secondary cell wall is composed of complex polymers that provide protective and structural properties to the cell wall. It includes four major biopolymers: hemicelluloses, cellulose, lignin and also pectin, but the major components are the first three (Balatinecz et al., 2001) (Figure 1.3A). The secondary cell walls are highly organized, with cellulose microfibrils deposited in S1, S2 and S3 layers. The three layers differ in the orientation of cellulose microfibrils (Figure 1.3 B) (Barnett and Bonham, 2004; Sticklen, 2008).

**Figure 1.3 Schematic diagrams of structure of secondary cell wall**

(A) Diagram of secondary cell wall relative to the primary wall and plasma membrane, which contains cellulose synthase enzymes as integral membrane proteins. (B) The secondary cell wall S1, S2 and S3 layers. Images were adapted from Sticklen (2008).

1.2.1 Biosynthesis of secondary cell wall components

Cellulose is a polymer of β-1, 4-linked glucose residues. Cellulose polymers are organized into cellulose microfibrils that are embedded in a network of hemicellulose and lignin. Cellulose is
synthesized by plasma membrane-bound enzyme complexes known as rosettes, which consist of cellulose synthases encoded by *CESA* (Cellulose Synthase) genes (Doblin et al., 2002). The Arabidopsis genome contains at least 10 *CESA* genes (Somerville, 2006). Some of them are involved in primary wall biosynthesis, such as *CESA1*, *CESA2*, *CESA3* and *CESA6*, while others are secondary wall associated *CESA4*, *CESA7* and *CESA8* (Somerville, 2006).

Lignin is an aromatic polymer with three-dimensional linkages, and consists of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, which are derived from *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, respectively. These are derived from cinnamic acid, the product of the reaction catalyzed by phenylalanine ammonia-lyase, via phenolic ring modification (hydroxylation and methylation) and a three-step reduction process catalyzed by 4-coumarate:CoA ligase, cinnamoyl-CoA reductase (CCR) and cinnamyl/sinapyl alcohol dehydrogenase (CAD/SAD). After biosynthesis of lignin monomers, they are transported to the cell wall, where they are oxidized by "polyphenol oxidases" such as peroxidase and laccases and then cross-coupled to the growing polymer to extend the complex three-dimensional lignin network (Boerjan et al., 2003). Most of the genes involved in the lignin biosynthetic pathway have been isolated and functionally characterized in Arabidopsis (Boerjan et al., 2003), but the exact roles of the polymerization enzymes have not been well defined *in planta*.

Hemicelluloses are a class of branched polysaccharides containing a variety of 5-and 6-carbon sugars. For example, xyloglucans are a major constituent of dicot primary walls, while arabinoxylan and glucomannans are the major hemicelluloses in secondary walls (Pauly and Keegstra, 2008). Several genes are known to take part in xylan biosynthesis (Liepman et al., 2005; Zhong et al., 2005), such as *FRA8* which encodes glycosyltransferase associated with xylan biosynthesis in Arabidopsis.

Although the biochemistry of secondary wall biosynthetic pathways has been extensively studied,
information on transcriptional regulators that control the secondary wall synthetic process is more limited. If the regulatory network controlling secondary cell wall synthesis can be well established, the cell walls of wood and plant fibers may be more easily manipulated.

1.3 Transcription factors associated with secondary cell wall formation

Recently, several transcription factors regulating secondary wall biosynthesis have been identified by genetic and reverse genetic analysis in Zinnia and Arabidopsis. These studies indicate that secondary wall–associated NAC domain protein1 (SND1), NAC secondary wall thickening promoting factor (NST1, NST2), vascular-related NAC-domain6 (VND6) and VND7 are key regulators of secondary wall biosynthesis in different cell types (Kubo et al., 2005; Mitsuda et al., 2005; Zhong et al., 2006; Yamaguchi et al., 2008). Besides these master switches, MYB transcription factors (MYB46, MYB83, MYB58 and MYB63) and a KNOX homeodomain protein (KNAT7), have been shown to act downstream of NAC domain transcription factors in regulating secondary wall biosynthesis (Zhong et al., 2008), and some were shown to be direct targets of NACs (Zhong et al., 2007; Zhong et al., 2008; McCarthy et al., 2009). KNAT7 is also a target of MYB46 (Ko et al., 2009). All these data suggest that a transcriptional network regulates the biosynthesis of three major components of secondary cell wall (lignin, cellulose and hemicellulose). A model for the network regulating secondary wall synthesis based on the above work is presented in Figure 1.4. This model presents a general framework for understanding the secondary cell wall transcriptional network, but it is still far from complete (Zhong et al., 2008).
1.4 Homeodomain proteins

Homeodomain proteins were originally discovered as the protein products encoded by homeotic genes and are characterized by a conserved sequence of 180bp, the homeodomain (Gehring et al., 1994a). The homeodomain was first identified in Drosophila homeotic genes, mutation in which result in homeotic (out of place) developmental phenotypes characterized by loss of segmental identity, for example leading to formation of a leg on the head of a fruit fly instead of the expected antenna (McGinnis et al., 1984). The homeodomain consists of three helices I, II and III, with helices II, III and the small turn connecting them forming a helix-turn-helix motif. The characteristic three-helix structure binds directly with conserved sequences in the target DNA or other proteins to form a complex involved in gene regulation (Gehring et al., 1994b).

1.4.1 KNOX family proteins

Plants contain several families of homeodomain containing transcription factors, such as HD-ZIP and other classes (Mukherjee et al., 2009). Plant Knotted-like homeobox (KNOX) proteins, called KNOTTED-LIKE ARABIDOPSIS THALIANA (KNAT) proteins in Arabidopsis, belong to the plant-specific three amino acid loop extension (TALE) superclass of homeodomain proteins.
(Hake et al., 2004). Distinguished from the other homeodomain proteins, the TALE superclass of homeodomain proteins has an extra three amino acids between helices 1 and 2 (Bertolino et al., 1995). KNOX proteins also have a KNOX (MEINOX) domain that forms an amphipathic helix having 9-13 turns with most of the conserved residues on one face of the helix and an ELK domain that forms two short helices with 24 amino acids (Figure 1.5).

The eight genes of the KNOX family in Arabidopsis can be grouped into two classes based on their sequence similarities and phylogenetic analysis of aligned sequences (Kerstetter et al., 1994). Class 1 KNOX proteins include SHOOTMERISTEMLESS (STM), BREVIPEDICELLUS (BP) / KNAT1, KNAT2 and KNAT6, while KNAT3, KNAT4, KNAT5 and KNAT7 belong to Class 2. Certain Class 1 KNOX proteins, which are mainly expressed in the shoot apex, are required for proper development of the shoot apical meristem (SAM). For example, the bp mutant shows reduced internode elongation and downward pointing pedicels (Douglas et al., 2002; Venglat et al., 2002). STM is essential for the initiation of the meristem during embryogenesis (Long et al., 1996). However, functions of Class 2 KNOX proteins are not clear. KNAT3, KNAT4 and KNAT5 are thought to participate in Arabidopsis root development based on their expression patterns in the Arabidopsis roots (Truernit et al., 2006). KNAT7 was identified as a transcription factor regulating secondary wall formation based on co-expression and mutant analysis (Brown et al., 2005; Persson et al., 2005), and reverse genetic analysis revealed an irregular xylem (irx) phenotype in the loss of function allele (Brown et al., 2005). Other researchers described a severe reduction in secondary wall thickening in fiber cells caused by an artificial dominant repression version of KNAT7 (Zhong et al., 2008). Our lab has found that KNAT7 is strongly upregulated over the course of inflorescence stem development associated with secondary wall formation (Ehlting et al., 2005) and that knat7 mutants are affected in both vessel and fiber wall formation (Li, 2008). Furthermore, KNAT7 acts as a transcriptional repressor when transiently expressed in Arabidopsis protoplasts (Li et al., submitted).
1.4.2 BELL family of homeodomain proteins

Arabidopsis BEL1-LIKE homeodomain (BLH) proteins also belong to the TALE class and like KNOX proteins, are unique to plants. In addition to the TALE motif in the homeodomain, BLH proteins have a conserved BELL domain and SKY domain. The BELL and SKY regions compose a conserved bipartite domain in the N terminus of BLH proteins called the MEINOX interacting domain (MID) (Balatinecz et al., 2001; Bellaoui et al., 2001; Müller et al., 2001). Some studies suggest that KNOX-BLH forms heterodimer transcription factor complexes by the interaction of the MEINOX and MID domains (Bellaoui et al., 2001; Müller et al., 2001). There are 13 BLH genes in Arabidopsis (Smith et al., 2004). BEL1 (BEL1) is the founding member of the group and is involved in ovule integument development as revealed by the phenotype of bell1 mutants (Reiser et al., 1995). The misexpression of BEL1-LIKE HOMEODOMAIN1 (BLH1) results in defective Arabidopsis embryo sac development (Pagnussat et al., 2007). The double mutant of the BEL1-like SAWTOOTH1 (SAW1) and SAW2 genes shows a phenotype of increased leaf serration. In addition, SAW1 and SAW2 negatively regulate the KNOX gene BP (Kumar et al., 2007). The Arabidopsis BLH protein BELLRINGER (BLR; it is also named as PENNYWISE, REPLUMLESS, VAAMANA) is required for inflorescence development (Byrne et al., 2003; Bao et al., 2004; Bhatt et al., 2004; Smith et al., 2004). In addition, BLR and its paralogous protein POUNDFOOLISH (PNF) play similar roles in shoot development and flower patterning, and exhibit functional redundancy in these roles (Yu et al., 2009). Moreover, the BLH protein ARABIDOPSIS THALIANA HOMEobox1 (ATH1) controls floral competency by activating FLOWERING LOCUS, a flowering repressor gene (Proveniers et al., 2007).
1.4.3 The interaction between KNOX and BLH proteins

The protein-protein interactions between KNOX and BLH family members are well characterized in different plant species. It is reported that the interactions between a potato KNOX protein, POTH1 (potato homeobox 1), and StBEL5 (Solanum tuberosum BEL5), which is a BEL1-like protein, directly repress ga20oxidase1 (ga20x1) promoter activity by binding to a specific promoter ga20x1 sequence (Chen et al., 2004). Also in maize, the interaction between KNOTTED1 (KN1) and a BLH protein, KIP (Knotted Interacting Protein) results in a heterodimer with increased affinity to the specific KNOX DNA-binding motif (Smith et al., 2002). In Arabidopsis, STM interacts with ATH1, PENNYWISE (PNY/BLH9) and POUNDFOOLISH (PNF/BLH8) to control the initiation and maintenance of floral meristems (Rutjens et al., 2009). PNY is reported to cooperate with KNAT1 to regulate inflorescence development (Smith and Hake, 2003). The BLH proteins SAW1 and SAW2 promote correct leaf shape by repressing BP expression in Arabidopsis leaves (Kumar et al., 2007). A survey of Arabidopsis TALE protein interactions using yeast two-hybrid assays suggests a network of TALE protein-protein interactions with each other and with OVATE FAMILY PROTEINS (OFPs; Hackbusch et al., 2005). It has also been shown that OFPs can regulate the activity of KNAT-BLH heterodimers: OFP5 suppresses the activity of a BLH1 - KNAT3 complex to promote normal development of the Arabidopsis embryo sac (Pagnussat et al., 2007).
1.4.4 Subcellular localization of KNOX-BLH complex

Interactions between KNOX and BLH proteins can affect cellular trafficking and localization of these transcription factors. The fusion protein STM-GFP and GFP-STM was excluded from the nucleus and remained in the cytoplasm when expressed in transgenic cells (Bhatt et al., 2004). The GFP-BLH3 fusion protein exerted a preference for the nuclear compartment, while it was also detected in cytoplasm (Cole et al., 2006). However, the ATH1/STM, BLH3/STM and PNY/STM heterodimers were all efficiently incorporated into the nuclear compartment (Cole et al., 2006). Similarly, PNY-GFP fluorescence was demonstrated to localize to the cytosol, while co-expressing PNY-GFP with KNAT1 or STM resulted in nuclear localization (Bhatt et al., 2004). Recently, other researchers found that ATH1-GFP and PNY-GFP alone localize to the cytosol and nucleus, while after heterodimerization of these BEL1-like proteins with STM they become completely nuclear localized (Rutjens et al., 2009).

1.5 Arabidopsis OVATE family proteins

Arabidopsis OVATE FAMILY PROTEINS (OFPs) were reported as novel transcriptional regulators that interact with TALE proteins by protein-protein interactions to form an interaction network (Hackbusch et al., 2005). OFPs contain a conserved C-terminal OVATE domain of approximately 70 amino acids. There are 18 OFP genes in Arabidopsis, and most of them contain a predicted nuclear localization signal but lack DNA binding domains (Hackbusch et al., 2005). Arabidopsis plants overexpressing OVATE FAMILY PROTEIN 1 (OFP1) and OFP4 display reduced aerial organ size and abnormal organ shapes (Wang et al., 2007; Li et al., submitted). OFP1 and OFP4 act as strong transcriptional repressors (Wang et al., 2007) and interact with KNAT7 both in yeast (Hackbusch et al., 2005) and in planta (Li et al., submitted). In addition OFP1 and OFP4 interaction with KNAT7 enhances the transcriptional repression activity of KNAT7 (Li et al., submitted). The ofp4 mutant and ofp1ofp4 double mutant display irregular xylem (irx) phenotypes similar to those found in knat7 (Li et al., submitted), and the OFP4 overexpression phenotypes are suppressed in a knat7 mutant, suggesting that a KNAT7-OFP4
complex is required both for aspects of secondary wall formation and other developmental processes. As mentioned above, a BLH-KNOX TALE complex containing OFP5 appears to be essential for normal development and cell specification in the Arabidopsis embryo sac (Pagnussat et al., 2007).

1.6 Summary of background data

KNAT7 was identified by expression profiling as a candidate transcription factor that could regulate secondary wall synthesis in xylem and interfascicular fibers during Arabidopsis inflorescence stem development (Ehlting et al., 2005). A poplar KNAT7 ortholog was identified and shows increased expression during secondary wall deposition during wood formation (Li, 2008). KNAT7 loss-of-function mutants show collapsed vessels in vascular bundles (Brown et al., 2005; Li, 2008), and increased interfascicular fiber cell wall thickness (Li, 2008), while KNAT7 overexpression lines under the control of the parsley 4CL1 promoter show decreased interfascicular fiber cell wall thickness (Li, 2008). Further work has shown that KNAT7 is the direct target of the SND1 master regulator (Zhong et al., 2008) and is positioned downstream of MYB46 (Ko et al., 2009), but KNAT7 targets are not known. As discussed above, selective interactions between KNOX and BLH proteins are required in some cases for site-specific DNA binding and for nuclear localization of the transcription factors. Although heterodimeric complexes between TALE homeodomain proteins regulating developmental processes in plants are well characterized, the majority of them involve Class 1 KNOX proteins, and the partners of Class 2 KNOX proteins are poorly understood. The study of Hackbusch (2005) presents a comprehensive analysis of TALE protein interactions, showing that KNAT7 and BLH5, BLH7, ATH1 interact specifically in vitro, and also interact with a subset of OFP proteins. BHL5 shows a similar expression pattern to KNAT7 in association with secondary cell wall deposition (Ehlting et al., 2005), but BLH6 and BLH10 expression is up-regulated within 6 hours of MYB46 induction, together with KNAT7 (Ko et al., 2009), suggesting that they could function together.
1.7 Research objectives

Although several BLH proteins have been shown to interact with KNAT7 *in vitro*, there is no information about the contribution of BLHs to secondary wall biosynthesis. Based on studies from previous papers and the work of our lab, we know that KNAT7 acts as a transcriptional repressor to regulate the secondary wall formation in coordination with OFPs. This study is focused on BLH1, BLH5, BLH6, BLH7, BLH10 and ATH1 as candidate members of a complex containing KNAT7 and OFPs, similar to the KNOX-BLH-OVATE complex recently shown to regulate egg cell development in Arabidopsis (Pagnussat et al., 2007). My research goal was to identify the possible member(s) of a KNAT7-BLH complex and investigate the function(s) of such potential KNAT7 interacting BLH partner(s).
Chapter 2. Identification of BLH members of a putative KNAT7-BLH-OFP complex regulating secondary wall formation in Arabidopsis

2.1 Introduction

This chapter presents a study aimed at identification of possible BEL1-LIKE HOMEODOMAIN (BLH) members of a hypothesized KNAT7-BLH-OFP complex involved in secondary cell wall formation. The Arabidopsis KNOX gene KNAT7 has been identified in expression profiling and other experiments as a member of a transcription factor network regulating secondary wall formation during xylem and fiber cell differentiation in Arabidopsis inflorescence stems (Ehlting et al., 2005; Zhong et al., 2008). knat7 mutants display an irregular xylem (irx) phenotype (Brown et al., 2005) as well as increased fiber wall thickness in Arabidopsis inflorescence stems (Li, 2008), suggesting defects in secondary wall composition. Yeast two hybrid assays showed that KNAT7 interacts with members of the Ovate Family Protein (OFP) transcription co-regulators (Hackbusch et al., 2005; Li et al., submitted), and the KNAT7-OFP1 and KNAT7-OFP4 interactions were confirmed by targeted yeast two hybrid assays and bimolecular fluorescence complementation analyses in planta (Li et al., submitted). This work also showed that interaction with OFP1 or OFP4 enhances KNAT7 transcriptional repression activity. Furthermore, an ofp4 mutant exhibits similar cell wall phenotypes as knat7, and the pleiotropic effects of OFP1 and OFP4 overexpression depend upon KNAT7 function (Li et al., submitted).

Co-expression analysis and yeast two hybrid analyses (Hackbusch et al., 2005) suggest that BEL1-LIKE HOMEODOMAIN (BLH) transcription factors could be part of a KNOX-BLH-OVATE transcription factor complex regulating aspects of secondary cell wall formation, together with KNAT7 and OFP1/4. In this chapter I used bioinformatic approaches to identify possible KNAT7 and/or OFP interacting BLH proteins and investigated the functional interactions through yeast two-hybrid and in planta bimolecular fluorescence complementation analyses. This work identified a BLH partner that specifically interacts with KNAT7.
2.2 Materials and methods

2.2.1 Phylogenetic analysis

Full-length amino acid sequences of 13 members of the Arabidopsis BLH family were downloaded from TAIR (The Arabidopsis Information Resource, http://www.arabidopsis.org/). The homologous sequences in other plants were identified by using BLAST (Basic Local Alignment Search Tool, Altschul et al., 1990) to search genome databases of Populus trichocarpa, Oryza sativa, Ricinus communis, and Physcomitrella patens at the JGI (Joint Genome Institute, http://genome.jgi-psf.org/poplar/poplar.home.html) and NCBI (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

An alignment of all amino acid sequences was created using MUSCLE v3.6 (Edgar, 2004) with default alignment parameters. The alignment was manually adjusted using Se-Al v2.0 (http://iubio.bio.indiana.edu/soft/iubionew/molbio/dna/analysis/Pist/main.html).

Three kinds of phylogenetic analytical methods were applied in this study: distance-based bootstrapping, Maximum Likelihood (ML) and Bayesian Inference (BI). To reconstruct the phylogenetic trees, PHYLIP v3.68 (Retief, 2000) was employed in distance-based analyses, RAxML v7.0.3 (Stamatakis, 2006) was used in ML analyses, while MrBayes v2.1.3 (Huelsenbeck and Ronquist, 2001) was used in the BI method. ProtTest v2.1 (Abascal et al., 2005) was conducted to test evolutionary models for the amino acid alignments.

In distance-based bootstrapping analyses, 500 replicates of bootstrap analysis were completed first, followed by 500 replicates of distance analysis with the JTT+G+I+F model. The consensus tree was generated by consensus calculating. In the ML analyses, the model of JTT+G+I+F selected by ProtTest was applied with 100 heuristic searches followed by 500 replicates of bootstrap analysis. In BI analyses the number of Markov chains was four. The number of generations was 10000, and the sampling frequency was one tree per 100 generations. The
substitution rate was set as GTR model invoking a gamma rate distribution and a proportion of invariant sites. The trees that were generated by three different methods were visualized using Tree View (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html), then exported as jpeg format before being imported into Microsoft Word (Microsoft Corporation).

2.2.2 Plant material and growth conditions

*Arabidopsis thaliana* (Arabidopsis) ecotype Columbia-0 was used as wild type throughout, and all mutants and transgenic lines are in this background. For seedlings used for phenotypic and genotypic analysis, seeds were surface-sterilized by 75% ethanol and grown on Murashige and Skoog (MS) Basal Salts with minimal organics (Sigma) and 1% sucrose, solidified with 0.7%(w/v) agar (Sigma). Seeds were cold-treated at 4°C in dark for 48 hours, then moved to 22°C under a 16/8 hr (light/dark) photoperiod and constant white light at approximately 120µmol m⁻² sec⁻¹ for seed germination and seedling growth for 7-10 days. For some experiments, seedlings were transferred into soil for further research.

For protoplast transfection, approximately 20 Col-0 seeds were germinated and grown in 2×2 inch pots containing a moist of Sunshine Mix #4 (SunGro Horticulture Canada Ltd, http://www.sungro.com) with 16/8 hr (light/dark) at approximately 120µmol m⁻² sec⁻¹ at 22°C. Leaves from 3-4 week old plants were used for protoplast isolation.

2.2.3 Generating split YFP and bait and prey of yeast two-hybrid constructs

Clones of the complete open reading frames of *BLH1, BLH5, BLH6, BLH7, BLH10, ATH1, KNAT7, OFP1* and *OFP4* were isolated from cDNA prepared from Arabidopsis inflorescence stem mRNA as previously described (Li, 2008). The clones of *BLH* candidates were transferred to the Gateway™ compatible destination vector pSAT4-DEST- n(174)EYFP-C1 (Citovsky et al., 2006) by LR-mediated recombination, to generate fusions to the N-terminal half of the yellow fluorescent protein (YFP). *KNAT7, OFP1* and *OFP4* were cloned into pSAT5-DEST-c(175-end)
EYFP-C1 (Citovsky et al., 2006) to generate fusions to the C-terminal half of YFP. BLH6 and BLH5 inserts were also cloned into pSAT6-EYFP-N1 (Citovsky et al., 2006) to generate C-terminal fusions to full-length YFP.

Truncated KNAT7 clones encoding different domains (KNOX1 domain, KNOX2 domain, MEINOX domain and homeodomain) were generated from a KNAT7 plasmid by PCR amplification. All the primers used in this study are summarized in Table 2.1. These clones were introduced into a Gateway™ entry vector pCR8 (Invitrogen). Each clone was transferred to Gateway™ compatible yeast two-hybrid bait and prey destination vectors (Invitrogen) by LR-mediated cloning. Clones of BLH6, BLH7, KNAT7, OFP1 and OFP4 open reading frames were introduced into yeast two-hybrid bait and prey vectors using the same strategy.

Table 2.1 Primer sequences to generate truncated KNAT7 clones for yeast two-hybrid assay.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH1:Gal4 DBD-KNAT7</td>
<td>ATGCAAGAAGCGGCACTAGG</td>
</tr>
<tr>
<td>ADH1:Gal4 AD-KNAT7</td>
<td>TTAGTGTTTGCCTTGACCTT</td>
</tr>
<tr>
<td>ADH1:Gal4 DBD-KNOX1</td>
<td>ATGCAAGAAGCGGCACTAGG</td>
</tr>
<tr>
<td>ADH1:Gal4 AD-KNOX1</td>
<td>CTAAGCGTAAGACGGAGAAG</td>
</tr>
<tr>
<td>ADH1:Gal4 DBD-KNOX2</td>
<td>ATGCGTTCTACGCTCCACAG</td>
</tr>
<tr>
<td>ADH1:Gal4 AD-KNOX2</td>
<td>TACCCTGCTCTCAAGTTGC</td>
</tr>
<tr>
<td>ADH1:Gal4 DBD-MEINOX</td>
<td>ATGCAAGAAGCGGCACTAGG</td>
</tr>
<tr>
<td>ADH1:Gal4 AD-MEINOX</td>
<td>TACCCTGCTCTCAAGTTGC</td>
</tr>
<tr>
<td>ADH1:Gal4 DBD-ELK-Homeodomain</td>
<td>ATGGAAAGAGTCAGACAAGAA</td>
</tr>
<tr>
<td>ADH1:Gal4 AD-ELK-Homeodomain</td>
<td>TTAGTGTTTGCCTTGACCTT</td>
</tr>
<tr>
<td>ADH1:Gal4 DBD-BLH6</td>
<td>ATGGAGATATTCACAGAAACACA</td>
</tr>
<tr>
<td>ADH1:Gal4 AD-BLH6</td>
<td>TCAAGCTCAAAAAATCATGTACCAA</td>
</tr>
<tr>
<td>ADH1:Gal4 DBD-OFP1</td>
<td>ATGGGTAAAATATCGTTAAGC</td>
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<tr>
<td>ADH1:Gal4 AD-OFP1</td>
<td>TATTTGGAATGGGGTGTTAAG</td>
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<td>ADH1:Gal4 DBD-OFP4</td>
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<td>ADH1:Gal4 AD-OFP4</td>
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<tr>
<td>ADH1:Gal4 DBD-BLH7</td>
<td>ATGGCCACTTTATCAAAACTGG</td>
</tr>
<tr>
<td>ADH1:Gal4 AD-BLH7</td>
<td>TCAAGCTCAAAAAATCATGCAACAA</td>
</tr>
</tbody>
</table>
2.2.4 Bimolecular Fluorescence Complementation (BiFC) assay in Arabidopsis mesophyll protoplasts
Arabidopsis leaf mesophyll protoplast cells were isolated and transfected as described previously (Wang et al., 2007). In brief, protoplasts were isolated from rosette leaves of 3-week-old plants. Constructs prepared as described above were transfected into protoplasts and incubated in the dark for 18-20 hr to allow expression of the introduced genes. The YFP fluorescence was examined and photographed using an Olympus AX70 light microscope.

2.2.5 Yeast two hybrid assay
The interaction between OFP1/OFP4/KNAT7 and BLH6/BLH7 were tested by using ProQuest Two-Hybrid System (Invitrogen) as described previously (Guo et al., 2009). OFP1, OFP4, KNAT7, KNOX1 domain, KNOX2 domain, MEINOX domain and Homeodomain were cloned into prey vector (pDEST22), and BLH6 (and BLH7 were cloned into bait vector (pDEST32). The known interaction between MYB75 and TT8 (Zimmermann et al., 2004a) was used as a positive control. Coexpression of BLH6 and BLH7 and the empty bait vector was used as a negative control. The ability of yeast transformants to grow on minimal SD (Synthetic Dextrose) medium lacking both leucine and tryptophan is indicative of the presence of both prey and bait constructs. Positive interactions were identified by their ability to activate HIS3, URA3, or LacZ genes, assayed by the appearance of yeast colonies on triple-selection minimal SD medium lacking leucine, tryptophan and uracil or histidine, or a by blue color when assayed with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

2.3 Results

2.3.1 Phylogenetic analysis of BELL family members in different plant species
Using amino acid sequences of thirteen members of the Arabidopsis thaliana BLH gene family as a query in BLAST searches, a set of 26 homologous proteins from other species was obtained,
including five homologous sequences from *Oryza sativa*, eighteen homologous sequences from *Populus trichocarpa*, two homologous sequences from *Physcomitrella patens* and one homologous sequence of *Ricinus communis*. These sequences, ranging in sizes from about 500 to 800 amino acids, are shown in Table 2.2 and were used as the 39 input sequences to generate phylogenetic trees.

Table 2.2 Species names and NCBI reference sequence numbers of the 39 BLH sequences included in this study.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Species</th>
<th>Gene Name</th>
<th>NCBI No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>At_BLH1</td>
<td>Arabidopsis thaliana</td>
<td>BLH (BEL1-LIKE_homeodomain 1)</td>
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<td>At_BLH2</td>
<td>Arabidopsis thaliana</td>
<td>BLH (BEL1-LIKE_homeodomain 2)</td>
<td>NP_001015797</td>
</tr>
<tr>
<td>At_BLH3</td>
<td>Arabidopsis thaliana</td>
<td>BLH (BEL1-LIKE_homeodomain 3)</td>
<td>NP_001017827</td>
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<tr>
<td>At_BLH4</td>
<td>Arabidopsis thaliana</td>
<td>BLH (BEL1-LIKE_homeodomain 4)</td>
<td>NP_001015404</td>
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<td>At_BLH5</td>
<td>Arabidopsis thaliana</td>
<td>BLH (BEL1-LIKE_homeodomain 5)</td>
<td>NP_180290</td>
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<tr>
<td>At_BLH6</td>
<td>Arabidopsis thaliana</td>
<td>BLH (BEL1-LIKE_homeodomain 6)</td>
<td>NP_001149116</td>
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<td>At_BLH7</td>
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<td>BLH (BEL1-LIKE_homeodomain 7)</td>
<td>NP_190325</td>
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<td>At_BLH8</td>
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<td>BLH (BEL1-LIKE_homeodomain 8)</td>
<td>NP_180868</td>
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<tr>
<td>At_BLH9</td>
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<td>BLH (BEL1-LIKE_homeodomain 9)</td>
<td>NP_180823</td>
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<td>At_BLH10</td>
<td>Arabidopsis thaliana</td>
<td>BLH (BEL1-LIKE_homeodomain 10)</td>
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<td>At_ATH3</td>
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<td>Os_4128929</td>
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<td>AAAS15263.1</td>
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<td>Os_0884427</td>
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<td>Pop_gw100121</td>
<td>Populus trichocarpa</td>
<td>predicted protein</td>
<td>NP_022320323</td>
</tr>
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</table>

Figure 2.1A shows the phylogenetic tree constructed using Bayesian Inference (BI) method with posterior probabilities indicated near the branches. The topology of this tree is similar to the tree
reconstructed by Maximum Likelihood (JTT+G+I+F model) shown in Figure 2.1B with bootstrap values indicated near the branches. Furthermore, the consensus tree (Figure 2.1 C) reconstructed using distance-based bootstrapping analyses with bootstrap values indicated near the branches is consistent with the BI and ML-JTT trees.

According to the alignments, the homologous sequences obtained from *Populus trichocarpa*, *Oryza sativa*, *Ricinus communis* and *Physcomitrella patens* contained a conserved SKY domain, BELL domain and Homeodomain (data not shown). This result suggests that they all belong to BLH family. It also points out that *BLH* genes are ancient, as not only vascular plants but also the moss *Physcomitrella* contains BLH homologs
Figure 2.1 Phylogenetic analyses of *BEL1*-like homeodomain proteins.

(A) Results of Bayesian analysis of *BEL1*-like homeodomain proteins. Posterior probabilities are indicated near branches. (B) Phylogenetic tree of *BEL1*-like homeodomain proteins constructed by ML (JTT model) using RAxML. Bootstrap values are indicated near branches. (C) Distance analysis of *BEL1*-like homeodomain proteins using PHYLIP. Bootstrap values are indicated near branches. BLAST searches were used to identify the homologous sequences from *Arabidopsis*, *Populus trichocarpa*, *Oryza sativa*, *Ricinus communis* and *Physcomitrella patens*.

See Materials and Methods for details.
2.3.2 *In silico* expression patterns of candidate BLH genes in *Arabidopsis*

Genes encoding Arabidopsis BLH proteins that are candidates for interaction with KNAT7 would be expected to share a common or overlapping expression pattern with *KNAT7*. In order to examine where the Arabidopsis BLH genes are expressed, and whether their expressions overlaps with the expression of *KNAT7* and OFPs, gene expression data from publicly available
microarray experiments was queried using both GENEVESTIGATOR v3 (Tomas et al., 2008); https://www.genevestigator.com/gv/index.jsp) and BAR (The Bio-Array Resource for plant functional genomics, http://bbc.botany.utoronto.ca/) sites for analysis. The data are summarized in Figure 2.2 and Figure 2.3.
Figure 2.2 Relative Arabidopsis BLH gene expression levels from anatomy in Genevestigator v3. Darker blue indicates higher expression.
Figure 2.2 shows a graphical summary of *in silico* analysis of relative expression levels of 13 members of *BLH* family genes as well as *KNAT7* and *OFP1* (there is no data for OFP4) in different organs, cells and developmental stages retrieved from the Anatomy expression set in GENEVESTIGATOR v3 site based on the data from 3110 Affymetrix ATH1 22K arrays. Many *BLH* genes have high transcript levels in seed coat, stem or the xylem and cork of hypocotyls, which overlap the expression pattern of *KNAT7*, such as *BLH5, BLH6, BLH7, BLH9* and *BLH10*. However the expression pattern of *BLH7* seems to be more general and widely distributed.

Figure 2.3 displays an *in silico* analysis of the expression levels of the 15 transcription factor genes in different organs from the “Developmental Map” in Arabidopsis eFP Browser at BAR. A comparison of the relative expression levels of 15 genes in Figure 2.3 shows that transcripts of *BLH1, BLH6* and *KNAT7* are more abundant in the second internode of stem than the first node, which indicates these genes are more highly expressed in stem regions undergoing secondary cell wall synthesis. *OFP1* and *KNAT7* are expressed at elevated levels in hypocotyls, and *BLH1, BLH6, BLH7, BLH9* and *ATH1* exhibit relatively high expression in this organ.
2.3.3 Bioinformatic and literature-based identification of potential KNAT7 interacting BLH proteins

According to a large-scale yeast two-hybrid assay (Hackbusch et al., 2005), BLH5, BLH7 and ATH1 interact with KNAT7 in yeast. BLH6 and BLH10 have been shown to be the direct targets of MYB46 together with KNAT7 (Ko et al., 2009), which suggests that BLH6 and BLH10 are also interesting candidates for KNAT7 interaction. **BHL5** shows a similar expression pattern to **KNAT7** in association with secondary cell wall deposition (Ehlting et al., 2005). Furthermore, BLH1 is the BLH protein that shows highest similarity to BLH5 (Roeder et al., 2003). In summary, the list of potential BLH components in a putative KNAT7-BLH complex includes BLH1, BLH5, BLH6, BLH7, BLH10 and ATH1.

2.3.4 BiFC screen for KNAT7 interacting BLH proteins

2.3.4.1 Subcellular localization of BLH candidates

KNAT7 nuclear localization has been reported by Zhong et al. (2008) and Li et al., (submitted). Recently, it has been shown that an ATH1-GFP fusion is found in both the cytosol and nucleus, while after heterodimerization with STM, a KNOX homeodomain protein, it becomes completely nuclear localized (Rutjens et al., 2009). BLH1 was shown to be located only in the nucleus but not in the nucleolus (Hackbusch et al., 2005). To test their subcellular localization, Arabidopsis protoplasts were transfected with C-terminal fusions BLH5 and BLH6 to full-length enhanced yellow fluorescent protein (EYFP). This analysis revealed that BLH5-F-EYFP and BLH6-F-EYFP fluorescence was primarily localized to the nuclei of transformed protoplasts, similarly to KNAT7-F-EYFP (Figure 2.4). However, some BLH6-F-EYFP fluorescence appeared to be present in the cytoplasm.
Figure 2.4 Subcellular localization of BLH candidates.

Top row, bright field images of representative protoplasts transfected with BLH5F-EYFP, BLH6-F-EYFP, KNAT7-F-EYFP under the control of the 35S promoter, and F-EYFP alone under the control of the 35S promoter. Bottom row, images of the same protoplasts obtained by imaging YFP fluorescence.

2.3.4.2 BLH6 interacts with KNAT7 in planta

Bimolecular Fluorescence Complementation (BiFC) (Hu et al., 2002) was used to assay protein-protein interactions between BLH candidates and KNAT7 in planta. The BLH candidates, KNAT7 and OFPs were fused to N or C terminal (N/C-EYFP) fragments of the enhanced yellow fluorescent protein (EYFP). The split EYFPs were fused to both the C- and N- termini of each gene (Figure 2.5). Neither of these fragments is capable of fluorescence alone. As controls, fusions of several proteins to full EYFP were made or were available. Different combinations of fusion constructs were used to transform Arabidopsis mesophyll protoplasts using an Arabidopsis leaf mesophyll protoplast transient expression system in order to test the ability of each BLH candidate to interact with KNAT7.
Figure 2.5 Schematic diagram representing two sets of BiFC constructs.

All the constructs are driven by cauliflower mosaic virus (CaMV) 35S promoter. Fusions of KNAT7, BLH6 and BLH5 to full EYFP were used as control. The BLH1, BLH5, BLH6, BLH7, BLH10 and ATH1 were fused to N-EYFP, while KNAT7, OFP1 and OFP4 were fused to C-EYFP. The difference between the two sets of constructs is the location of split EYFP. The “+” means the cotransformation of Arabidopsis mesophyll protoplasts with BLH candidates and KNAT7, or BLH6 and OFP1/OFP4.

Representative positive and negative data are shown in Figure 2.6. Among all BLH candidates (BLH1, BLH5, BLH6, BLH7, BLH10 and ATH1) tested for interaction with KNAT7 in different combinations, only the co-expression of KNAT7: C-EYFP with BLH6:N-EYFP or C-EYFP:KNAT7 with N-EYFP:BLH6 generated nuclear localized fluorescence (Figure 2.6). Interestingly, the BLH6 paralog BLH7 (Figures, 2.1 A, B, C) showed no evidence of interaction with KNAT7 (data not shown), suggesting a high level of specificity in BLH-KNAT7 interactions. In addition, the interaction between BLH6 and OFP4/OFP1 was tested, but the interaction was undetected by BiFC (data not shown).
Figure 2.6 BLH6 interacts with KNAT7 *in planta*

Left column, bright field images of representative protoplasts co-transfected with
KNAT7-C-EYFP and BLH6-N-EYFP, KNAT7-C-EYFP and BLH5-N-EYFP, EMPTY-C-EYFP
and BLH6-N-EYFP, MEINOX Domain-C-EYFP and BLH6-C-EYFP. Right column, images of
the same protoplasts obtained by imaging YFP fluorescence.

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2.3.4.3 BLH6 interacts with the KNAT7 MEINOX domain

While BLH and KNOX are both plant specific TALE homeodomain proteins, BLH and KNOX
proteins differ greatly in the N terminal regions (Bharathan et al., 1997). There is a MEINOX
domain at the N terminus of KNOX proteins characterized by an amphipathic helix containing
9-13 turns, with most of the conserved residues on one face of the helix (Bharathan et al., 1997).
An ELK-HD (Homeodomain) domain at the C-terminus of KNOX proteins is highly conserved
within the family and folds into three α-helices. The heterodimers formed by BLH and KNOX
proteins investigated to date require the interactions between MEINOX domain of KNOX
proteins and the SKY and BELL domains upstream of homeodomain of BLH proteins (Mukherjee and Bürglin, 2007; Hay and Tsiantis, 2009; Mukherjee et al., 2009).

BiFC was used to test the interaction between the KNAT7 MEINOX domain with BLH6 in planta. Figure 2.6 reveals that coexpression of BLH6: N-EYFP and KNAT7 MEINOX Domain: C-EYF in protoplasts resulted in fluorescence complementation, indicating interaction of BLH6 with the KNAT7 MEINOX domain. While fluorescence was mainly in the nucleus, it was weak compared to fluorescence generated by BLH6: N-EYFP and KNAT7:C-EYFP interaction, which was strongly localized to the nucleus (Figure 2.6). The difference between the BiFC fluorescence signals was more apparent after increasing the brightness of images, shown in Figure 2.7. While the KNAT7- F-EYFP and KNAT7: C-EYFP with BLH6: N-EYFP was still only observed in nuclei in these images, the weaker fluorescence generated by the interaction of BLH6: N-EYFP and MEINOX Domain: C-EYFP was clearly detected in the cytoplasm as well, and BLH6-F-EYFP generated fluorescence was also observed outside of the nucleus.

These results suggest that BLH6 requires interaction with KNAT7 to direct exclusive nuclear localization. Since two KNOX nuclear localization signal (NLS) sequences are found in the ELK-HD domain of KNOX proteins (Meisel and Lam, 1996), the KNAT7 MEINOX domain itself may be poorly targeted to the nucleus, resulting in poor nuclear localization of the KNAT7 MEINOX-BLH6 complex.
Figure 2.7 BLH6 interacts with MEINOX domain of KNAT7 in planta

Top row, bright field images of representative protoplasts transfected with BLH6-F-EYFP, KNAT7-F-EYFP under the control of the 35S promoter. Co-transfected with MEINOX Domain-C-EYFP and BLH6-N-EYFP, KNAT7-C-EYFP and BLH6-N-EYFP. Bottom row, images of the same protoplasts obtained by imaging YFP fluorescence with increased brightness.

2.3.5 Yeast two hybrid assay of BLH- and OFP-KNAT7 interaction candidates

The interactions between BLH6 and KNAT7/OFP1 were not reported in a large-scale yeast two-hybrid assay (Hackbusch et al., 2005), but BLH6 was reported to interact with OFP4. As BLH6 was the only interacting partner of KNAT7 selected from BiFC screening, these interactions were tested again using our yeast two-hybrid system. As BLH6 is paralogous to BLH7, the interactions between BLH7 with KNAT7, OFP1 and OFP4 were also tested by Y2H even though we did not find any interactions between BLH7 and KNAT7 in the BiFC assay. KNAT7, BLH6, BLH7, OFP1, and OFP4 DNA binding (DB) and activation domain (AD) fusion protein constructs were generated. The ability of co-transformed yeast strains to activate expression of the URA3 nutritional marker gene and the LacZ gene was tested using a growth assay on drop-out media (Ura-) or by the generation of blue color using X-gal (5-bromo-4-chloro-3-indolyl-β-D- galactopyranoside) as the substrate for the LacZ gene, to test for protein-protein interactions. The data presented in Figure 2.8 shows that BLH6 interacts with
KNAT7, but not with OFP4 or OFP1, and that there was no detectable interaction of BLH7 with KNAT7, OFP1 or OFP4.

**Figure 2.8 Yeast two-hybrid assay of BLH6-, or BLH7- and OFP-KNAT7 interaction candidates.**

Assay of AD-KNAT7, AD-OFP1, AD-OFP4 interaction with BD-BLH6, or BD-BLH7 using two reporter genes, *URA3* (assayed in Ura⁻ media) and *LacZ* (assayed using X-gal), with growth controls in Leu⁻ Trp⁻ media. MYB75-TT8 interaction was used as a positive control, and BD-BLH –empty prey vector (AD-) interaction was used as a negative control.

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**2.3.5.1 BLH6 interacts with the MEINOX domain of KNAT7**

Yeast two hybrid system was used to test the function of the KNAT7 MEINOX domain in interaction with BLH6, as predicted by work on other KNOX proteins (Burglin, 1997; Mukherjee and Bürglin, 2007) and from BiFC results (Figures 2.6 and 2.7). Four KNAT7 domains, KNOX1, KNOX2, MEINOX (KNOX1 and KNOX2), and the homeodomain were fused to the activation domain (AD) in yeast two-hybrid vectors, and tested for their abilities to interact with a BD (DNA binding domain) -BLH6 fusion (Figure 2.9B). The KNAT7 KNOX2 and MEINOX domains, but no other domains, were able to interact strongly with BLH6, suggesting that the KNOX2 portion of the MEINOX domain is sufficient for KNAT7 interaction with BLH6.
proteins.

**Figure 2.9 BLH6 interacts with the MEINOX domain of KNAT7.**

(A) Schematic diagram of KNAT7 with four different fragments, KNOX1, KNOX2, MEINOX (KNOX1+KNOX2) domain and Homeodomain. (B) Assay of AD-KNAT7 fragments interaction with BD-BLH6 using two reporter genes, *URA3* (assayed in Ura⁻ media) and *LacZ* (assayed on X-gal membrane), with growth controls in Leu⁻ Trp⁻ media. MYB75-TT8 interaction was used as a positive control, and BD-BLH6 –empty prey vector (AD-) interaction was used as a negative control.
2.3.5.2 OFP4 interacts with the homeodomain of KNAT7

To gain further insight into the nature of a putative KNAT7-BLH6-OFP complex, the ability of KNAT7 to interact with OFP1 and OFP4, earlier reported by Hackbusch et al. (2005), was re-tested using our yeast two-hybrid system. Figure 2.10 indicates that KNAT7 fused to DNA-binding domain (BD) interacts with OFP4 fused to Activation Domain (AD) in yeast, based on growth on His’ and Ura’ selective media, comparable to that of the MYB75-TT8 interaction (Zimmermann et al., 2004b) used as a positive control. Using similar criteria, detectable but weaker interaction (growth on His’ but not Ura’ selective media) was found between KNAT7 and OFP1. Neither interaction resulted in detectable β-galactosidase activity, using *LacZ* gene expression as a read-out, in contrast to the MYB75-TT8 interaction (not shown).

**Figure 2.10 KNAT7 interacts with OFP1 and OFP4**

Assay of BD-KNAT7 interaction with AD-OFP1 and AD-OFP4 using two reporter genes, *URA3* (assayed on Ura’ media) and *HIS3* (assayed in His’ media), with growth controls in Leu’ Trp’ media. Growth on His’ media and absence of growth on Ura’ media is indicative of a weak protein-protein interaction. MYB75-TT8 interaction was used as a positive control, and BD-KNAT7 – AD empty vector interaction was used as a negative control.
It was demonstrated that the protein-protein interaction between certain KNOX proteins and OFP is mediated by homeodomain and ovate domain, respectively (Hackbusch et al., 2005). To confirm this directly for the KNAT7-OFP1 and –OFP4 interactions, the set of four fusions of KNAT7 truncated domains (KNOX1, KNOX2, MEINOX, and Homeodomain) fused to the GAL4 DB was employed and cotransformed with AD-OFP1 and –OFP4 fusions (Figure 2.11). The KNAT7 homeodomain, but no other domain, was able to interact with OFP4 and showed a weak interaction with OFP1, suggesting that the homeodomain is sufficient for KNAT7 interaction with OFP proteins.

**Figure 2.11 OFP4 interacts with the homeodomain of KNAT7**

Assay of BD-KNAT7 fragments interaction with AD-OFP1 and AD-OFP4 using two reporter genes, *URA3* (assayed on Ura⁻ media) and *HIS3* (assayed in His⁻ media), with growth controls in Leu⁻ Trp⁻ media. Growth on His⁻ media and absence of growth on Ura⁻ media is indicative of weak protein-protein interactions. MYB75-TTA8 interaction was used as a positive control. The positive and negative controls are shown in Figure 2.10.
2.4 Discussion

In this chapter, I tested the hypothesis that KNAT7 interacts with one or more BLH proteins, as part of a putative KNAT7-BLH-OFP complex that could play a role in the regulation of secondary wall biosynthesis. In order to identify potential Arabidopsis BLH proteins as candidates for interaction with KNAT7, I first examined the structure of the BLH gene family in Arabidopsis and other plant species with fully sequenced genomes using phylogenetic methods. Homologs of Arabidopsis BLH genes were found in poplar, rice, and Physcomitrella, and their inferred phylogenetic relationships to Arabidopsis BLH paralogs were consistent among three phylogenetic trees generated using different methods. This result indicates they are related by common ancestry, and homologous genes in other species are likely to share the same function or activity as the Arabidopsis genes. Considering the functions of some Arabidopsis BLH genes such as BEL1, ATH1 and BLH1 have been already fully documented (Reiser et al., 1995; Byrne et al., 2003; Roeder et al., 2003; Bhatt et al., 2004; Kumar et al., 2007; Pagnussat et al., 2007; Proveniers et al., 2007; Yu et al., 2009), the apparent orthologous genes in poplar will probably share these functions. For example, Pop_gwIII246 and Pop_gw8865 appear orthologous to AtBEL1, and may have AtBEL1 function, but have been duplicated in the poplar lineage, consistent with the whole genome duplication event specific to the Salicaceae (Tuskan et al., 2006). At_ATH1 also has two duplicated orthologous genes, Pop_II000155 and Pop_estVI0194. In addition, BLH1 has two apparent poplar orthologs Pop_estVI1097 and Pop_gw1XVI12211, as well as one from *Ricinus communis* (Rc_223536621).

Based on the phylogenetic trees shown in Figures 2.1 A, B and C, several pairs of Arabidopsis BLH paralogs are evident, including At_BLH8 and At_BLH9, At_BLH4 and At_BLH2, At_BLH7 and At_BLH6, At_BLH10 and At_BLH3. This result is consistent with a previous study (Kumar, 2006), except that in the neighbour joining tree in that study, At_BLH5 was inferred to be paralogous to At_BLH1. With the exception of At_BLH10 and At_BLH3, each of these Arabidopsis paralog pairs has potential poplar orthologs, suggesting the potential for
conserved function in these two species. My analysis indicates that At.BLH5 does not have any paralogs or apparent orthologs in poplar, suggesting the possibility of a lineage-specific function for At.BLH5.

Based on the in silico analysis of relative expression levels, some of the 13 members of the Arabidopsis BLH gene family have similar expression patterns as KNAT7, including BLH1, BLH5, BLH6, BLH7, BLH9 and BLH10, especially in the second internode of stem and the xylem and cork of hypocotyls, which contain tissues with the cells undergoing secondary cell wall formation. More research in the future will be needed to understand the expression patterns of candidate BLH genes, for example, using promoter: GUS fusion constructs to track gene expression levels in specific tissues.

Based on these results, I tested a number of potential BLH-KNAT7 interactions by BiFC and yeast two hybrid analyses. BLH6 was the only interacting partner identified from these analyses. Even though the protein sequence of BLH7 shares 68% similarity with BLH6, the BLH7-KNAT7 interaction was undetectable in planta and in yeast. Also, BLH7 did not interact with OFP1 in yeast, which is consistent with results reported by Hackbusch et al. (2005) in planta. However, it is possible that BLH7 could act redundantly with BLH6 because of their overlapping expression patterns and sequence homology. In total, I could not confirm several of the protein-protein interactions predicted by Hackbusch et al. (2005) on the basis of a large screen of KNOX, BLH, and OFP proteins, while I identified new interactions not detected in that study. This points out the need to more thoroughly test individual protein-protein interactions predicted from large-scale screens.

It has been previously described that the MEINOX domain of STM and the BELL domain of BLH3 are sufficient for interaction in vitro and in planta (Cole et al., 2006). Both the BiFC and yeast two-hybrid data revealed that MEINOX domain of KNAT7 is essential and sufficient for
interaction with BLH6. If the MEINOX domain provides a central backbone for an assembly of different protein complexes in plants, such functional constraints could easily explain its conservation during evolution. The homeodomain in plant KNOX proteins has been shown to contain two functional NLS sequences (Meisel and Lam, 1996). These NLS seem to be essential for localization of the heterodimer formed between KNAT7 and BLH6, as the interaction between MEINOX domain and BLH6 were not only observed in the nucleus but also cytoplasm (Figures 2.6 and 2.7). While the interaction between KNAT7 and BLH6 was only detected in the nucleus, BLH6 alone showed a less specific pattern of nuclear localization (Figures 2.4 and 2.7). Taken together, these data suggest that efficient targeting of the KNAT7-BLH6 complex to the nucleus requires the KNAT7 interaction partner.

I verified KNAT7-OFP4 and KNAT7-OFP1 interactions previously identified by BiFC (Li et al., submitted) by using yeast two-hybrid assays, but found only weak interaction between KNAT7 and OFP1. In addition, my data show that the KNAT7 homeodomain seems to mediate the interaction of KNAT7 with OFP4. However, in terms of BiFC assay carried out in Arabidopsis leaf mesophyll protoplasts, KNAT7-OFP1 and KNAT7-OFP4 interactions appeared equally strong (Li et al., submitted). In addition, both OFP1 and OFP4 were able to enhance KNAT7 repression activity in the protoplast system (Li et al., submitted), consistent with observation that the phenotypes of OFP4 and OFP1 overexpression mutants are dependent on KNAT7 function. It is possible that there is another member of this complex, such as BLH6, present in protoplasts, stabilizing OFP-KNAT7 interactions in vivo, thus resulting in stronger OFP1-KNAT7 in planta interaction than observed in yeast.

Based on the protein-protein interaction data presented in this chapter, and supporting data from Li et al. (submitted) on OFP1/4-KNAT7 interaction, a model for KNAT7-OFP-BLH6 complex can be proposed as shown in Figure 2.14. As BLH6 interacts with the KNAT7 MEINOX domain and OFPs interact with its homeodomain, KNAT7 may act as a scaffold that allows for
interaction with both BLH6 and OFP partners, without direct BLH6-OFP interaction as found in our *in planta* and *in vitro* assays. Further research is needed to confirm the existence of the complex, such as yeast three-hybrid assay, immunoprecipitation, and/or pull-down assays.

**Figure 2.12 Proposed KNAT7-OFP-BLH6 complex model regulating secondary cell wall.**

KNAT7 could act as a bridge connecting BLH6 and OFPs. The interaction between KNAT7 and BLH6 is mediated by MEINOX Domain, while OFPs interact with KNAT7 through Homeodomain.
Chapter 3. Investigation of Arabidopsis BLH function in secondary wall formation

3.1 Introduction
Arabidopsis BEL1-LIKE homeodomain (BLH) proteins belong to the TALE class of homeodomain proteins which are plant specific proteins. There are 13 BLH genes in Arabidopsis (Smith et al., 2004). The functions of some of them, such as BEL1, BLH1, SAW1, SW2, BLR, PNF and ATH1 have been reported by different groups (Reiser et al., 1995; Byrne et al., 2003; Roeder et al., 2003; Bhatt et al., 2004; Kumar et al., 2007; Pagnussat et al., 2007; Proveniers et al., 2007; Yu et al., 2009).

Although the expression of certain BLH genes appears relatively high in the stem, xylem and cork of hypocotyls (Chapter 2), the functions, if any, of BLHs in secondary cell wall formation are poorly understood. In light of expression profiling (Ehlting et al., 2005) and protein-protein interaction data (Chapter 2), BLH5 and BLH6 were selected as our priority interaction partners with KNAT7 that participate in a KNAT7-BLH-OFP complex. In this study, the goal was to test whether BLH5 or BLH6 play roles in secondary cell wall formation by examining the phenotypes of loss-of-function mutants.

3.2 Material and methods

3.2.1 Plant material and growth conditions
Growth conditions for Arabidopsis thaliana ecotype Columbia-0 and T-DNA insertion lines in this background were as described in Section 2.2.2. Regions of 5cm from the bottom part of inflorescence stems from plants were approximately 6-8 week old were used for phenotypic characterizations.
3.2.2 T-DNA and reverse transcription PCR

T-DNA insertion mutant lines for BLH proteins were obtained from the Arabidopsis Biological Resource Center (http://Arabidopsis.org). The presence of the T-DNA insertion was examined by PCR using gene specific primer (GATCATGCTAGCAAGACAAACG and TGAAGAATTATCCGGTTCTG for blh5 and TCAATGGGGCTATAAGCCTG and TTGGGTTACGTTTTGTTTTCAG for blh6) and T-DNA left border LBa1 (TGGTTTCAGTAGTGGGCCATCG). Lines were genotyped to select homozygotes at the insertion site by PCR amplification using a combination of gene specific primers and T-DNA primer and transcriptional levels were tested by semi-quantitative RT-PCR using oligos (ATGGGCTGCTTTCTTTCTTGGA and CTAATCCATGATTTGATAAGT for blh5 and ATGGAGAATTATCCAGAAACA and TCAAGCTACAAAATCATGTACC for blh6) spanning the insertion sites.

Total RNA was extracted from 3-week-old rosette leaves or 6-week-old stems of Arabidopsis plants using the RNeasy plant mini kit (Qiagen). Single-strand cDNAs were synthesized via reverse transcription using Omniscript RT reverse transcriptase kit (Qiagen).

3.2.3 Generation of 4CL1:BLH constructs

Full-length cDNA clones of the complete open reading frames of BLH5 and BLH6 with BglII and PstI sites at 5’ and 3’ ends were isolated from Arabidopsis cDNA. The products were subsequently digested with BglII and PstI and ligated into the binary vector pSM-2 (Canam et al., 2006) containing the vascular specific 4CL1 (Petroselinum crispum 4-Coumarate:CoA ligase1) promoter (Hauffe et al., 1991). The binary vectors containing 4CL1:BLH5 and 4CL1:BLH6 were confirmed by sequence analysis.

3.2.4 Arabidopsis transformation

All transgenic lines were generated by transformation of Arabidopsis Columbia wild type using
the Agrobacterium tumefaciens-mediated floral dip method (Clough and Bent, 1998). Single colonies of Agrobacterium strain GV301 containing a binary vector with the proper construct were inoculated into 5 ml LB (Luria-Bertani) medium containing proper antibiotics and grown overnight at 28°C, then transferred to 250 ml of LB medium containing the antibiotics and shaken at 28°C overnight. The culture was spun for 20 minutes at 4°C in a SORVALL RC-5C centrifuge using a GS-3 rotor at 3000 rpm. The pellet was resuspended with infiltration medium. The plants were dipped into the medium for 5 minutes and covered with a plastic bag and stored horizontally overnight at room temperature in the dark. The plants were transferred to long-day conditions to allow seed maturation. Seeds were harvested and sown onto MS medium containing 50 µg/ml hygromycin. The healthy seedlings (T₁) were planted into soil and grown under long-day condition to generate next generation (T₂). The expression levels in T₁ individuals were determined by semi-quantitative RT-PCR. T₂ seeds were selected on MS medium containing 25 µg/ml hygromycin, homozygotes were picked depending on the ratio of resistant to susceptible individuals.

3.2.5 Toluidine blue staining
Fresh inflorescence stem tissues were hand sectioned with the use of the razor blades. Sections were stained directly on the slide in a drop of aqueous 0.02% toluidine blue O (Sigma) for 1 minute, rinsed in water and mounted in a drop of 50% glycerol beneath a coverslip and examined immediately with an Olympus AX70 light microscope.

3.2.6 Phloroglucinol-HCL staining
Hand sections of fresh 6-week-old Arabidopsis stems were stained with 1% phloroglucinol (w/v) in concentrated HCl for 5 min and mounted in a drop of 50% glycerol beneath a coverslip and observed and photographed immediately under Olympus AX70 light microscope.
3.2.7 Mäule staining

Hand sections of fresh 6-week-old Arabidopsis stems were treated for 10 min with 0.5% KMnO₄ and rinsed in water. Then sections were treated with 30% HCl until the brown colour disappeared, rinsed in water, mounted in concentrated NH₄OH, and examined under Olympus AX70 light microscope.

3.2.8 Generation of a GD-BLH6 construct

The full-length open-reading frame (ORF) of BLH6 was amplified by PCR using Arabidopsis cDNA with ClaI sites at 5’ and 3’ ends. The PCR fragment was then digested with ClaI and cloned in frame with an N-terminal Gal4 Binding Domain (GD) tag into the pUC19 vector under the control of the double 35S enhancer promoter of CaMV (Wang et al., 2007). The vector containing GD-BLH6 was confirmed by sequence analysis.

3.2.9 Protoplast transfection assays

Arabidopsis leaf mesophyll protoplast cells were isolated and transfected as described in section 2.2.4. Transactivators LD-VP16 and GD-VP16, reporters LexA(2x)-Gal4(2x)GUS and Gal4-35S:GUS were obtained from Dr. Shucai Wang, UBC (Wang et al., 2007). All reporter and effector plasmids used in transfection assays were prepared using the EndoFree Plasmid Maxi Kit (Qiagen). Ten μg of effector and reporter plasmid DNA described above were transfected into protoplasts and incubated in the dark for 20-22 hr. After incubation, cells were centrifuged at 180g for 3 min, and the supernatant was removed. The cells were resuspended in 100 μL 1X cell culture lysis reagent (Promega Corp., Madison, WI; Cat #153A) and immediately followed with MUG (4-methylumbelliferyl-β-D-glucuronide) assay as described previously (Wang et al., 2007). All transfection assays were performed as three replicates, and assays were repeated on at least two separate occasions.
3.3 Results

3.3.1 Test of BLH5 function

3.3.1.1 Identification of blh5 mutant

Based on analysis of expression profiling data (Ehlting et al., 2005) which showed strong co-expression of BLH5 and KNAT7, BLH5 was our top candidate for a BLH component of a KNAT7-BLH heterodimer complex at the outset of my work. In order to test the function of BLH5 in secondary wall biosynthesis, I used a reverse genetics approach. I obtained the BLH5 T-DNA insertion line SALK_122693, from the Salk Institute. The T-DNA insertion site was confirmed by PCR with T-DNA border flanking sequences primers. After generation of homozygous plants for the insertion, the T-DNA insertion site was verified by sequencing. The insertion was located within the second exon of BLH5. According to the results of semi-quantitative RT-PCR, the expression of BLH5 is undetectable in plants homozygous for the SALK_122693 allele (blh5-1) (Figure 3.1). This suggests that plants homozygous for this blh5 allele are loss of function mutant allele of BLH5. The plants homozygous for the blh5-1 allele were the material for all further experiments.

Figure 3.1 Characterization of plant loss of RNA expression of BLH5

Semi-quantitative RT-PCR analysis of BLH5 expression in blh5-1 and in wild type. No transcript was detected in the blh5-1 mutant. ACTIN2 was used as a reference control.
3.3.1.2 Phenotypic characterization of \textit{blh5} mutant

I examined the morphology and inflorescence stem anatomy of the \textit{blh5-1} loss-of-function mutant. Anatomy at the base inflorescence stems from 6-8 week old plants were analyzed by staining with toluidine blue and phloroglucinol-HCL (Figure 3.2). The \textit{blh5-1} mutant had normal phenotypes indistinguishable from wild type, with no differences in vessel or fiber cell wall thickness, or staining observed. This suggests that BLH5 by itself it does not play a strong role in secondary wall formation, but functional redundancy with other \textit{BLH} genes is a possibility.
Figure 3.2 Phenotypic characterizations of loss-of-function mutants of BLH5

Cross sections of the bottom of florescence stem of 6-week-old blh5-1 plants (A, C, E), wild type (Col-0) (B, D, F). Phloroglucinol-HCL staining of cross sections (A, B). Toluidine blue staining of cross sections (C-F). The phloroglucinol-HCL reagent detects aldehyde groups contained in lignin and results in red staining that is generally indicative of the presence of lignin. Bars, 5 mm.

3.3.1.3 Over-expression of BLH5

Considering there was no phenotype of blh5-1 loss-of-function mutant, 4CL1:BLH5 overexpression lines were generated. The 4CL1 promoter drives xylem-localized expression
(Hauffe et al., 1991), and 4CL1: KNAT7 expressing lines have a distinct phenotype, opposite that of knat7 (Li, 2008). Seeds of T₀ 4CL1: BLH5 overexpression plants were selected in hygromycin plates, and resistant plants were transferred to soil. T₁ plants were checked for expression of BLH5, and a line with the highest expression was selected for analysis (Figure 3.3). Homozygote 4CL1: BLH5 T₂ plants were identified for phenotypic analysis.

**Figure 3.3 Expression of BLH5 in overexpression lines**

Semi-quantitative RT-PCR analysis of BLH5 expression in BLH5 overexpression Line 1, Line 2 and in wild type. Highest transcript was detected in Line 1. ACTIN2 was used as a reference control.

I examined the morphology and inflorescence stem anatomy of BLH5 overexpression mutant as described above. Figure 3.4 shows representative results from examination of multiple plants, and indicates that the 4CL1: BLH5 mutant had normal phenotypes and was indistinguishable from wild type.
Figure 3.4 Anatomical characterization of 4CL1:BLH5 mutants.

Images show sections of bottoms of fresh stems of 4CL1:BLH5 (A, C, E) and wild type Col-0 (B, D, F) for analysis of general anatomy during secondary cell wall formation. Phloroglucinol-HCL staining of cross sections (A-D). Toluidine blue staining of cross sections (E, F). Bars, 5 mm.
3.3.2 Phenotypic characterization of \textit{blh6} mutant

3.3.2.1 Identification of a \textit{blh6} mutant

The second \textit{BLH} candidate analysis was \textit{BLH6} and T-DNA insertion mutant lines were obtained from the SIGnal database (http://signal.salk.edu). Among the T-DNA insertion lines available, line Salk_011023, was the only one with an exon insertion, and therefore was chosen for further analysis. Lines homozygous for the T-DNA insertion were selected by PCR. The T-DNA insertion site in the last exon was confirmed by sequencing. Semi-quantitative RT-PCR showed that the expression of \textit{BLH6} was undetectable in the homozygous plants, and the allele was named \textit{blh6-1} (\textit{blh6}) (Figure 3.5). These results suggest that \textit{blh6-1} is a loss of function mutant of \textit{BLH6}. Plants homozygous for \textit{blh6} were used as the material for all subsequent experiments.

\textbf{Figure 3.5 Characterization of the \textit{blh6-1} allele}

Semi-quantitative RT-PCR analysis of \textit{BLH6} expression in \textit{blh6-1} and in wild type. No transcript was detected in the \textit{blh6-1} mutant. \textit{ACTIN2} was used as a reference control.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{blh6-1-allele.png}
\caption{Semi-quantitative RT-PCR analysis of \textit{BLH6} expression in \textit{blh6-1} and in wild type.}
\end{figure}

3.3.2.2 Phenotypic characterization of the \textit{blh6} mutant

I examined the morphology and inflorescence stem anatomy of the \textit{blh6} loss-of-function mutant. Anatomy at the bases of inflorescence stems from 6-week-old plants from each genotype was analyzed by staining with toluidine blue, phloroglucinol-HCL, and Mäule staining, with representative results shown in Figure 3.6. The phloroglucinol-HCl reagent gives a red reaction.
when it reacts with aldehyde groups in the lignin polymer, whereas the Mäule reagent gives a qualitative indication of lignin monomer composition by staining G units in yellow and S units in red. Compared with the wild type, the secondary walls of interfascicular fibers in blh6 mutants appeared slightly thicker than those of WT, especially when viewed by Mäule and toluidine blue staining (Figure 3.6 A, E). The blh6 mutant also exhibited stronger red staining with the Mäule reagent in interfascicular fiber cells (Figure 3.6 A), suggesting that S unit deposition may be increased in these cells. Further chemical analysis of total lignin and G and S lignin units in these mutants is needed to confirm this phenotype, as well as more detailed analysis of cell wall thickness by electron microscopy. In contrast to the knat7 and ofp4 mutants, no irx phenotype was observed in the blh6 mutant.
Figure 3.6 Anatomical characterization of the \textit{blh6} loss-of-function mutant.

Images show cross sections of internodes at the bases of 6-week-old plants. \textit{blh6-1} (A,C,E), wild type Col-0 (B,D,F). Mäule staining of cross sections (A, B). Phloroglucinol-HCL staining of cross sections (C, D). Toluidine blue staining of cross section (E, F). Green arrows indicate the slightly thicker cell wall in \textit{blh6-1} mutant. Bars, 5 mm.

3.3.2.3 Transcriptional activity of BLH6

Previous data showed that OFP1, OFP4 and KNAT7 all function as transcriptional repressors in protoplast transfection assays (Wang et al., 2007; Li et al., submitted). Since BLH6 interacts with
KNAT7 in vivo (Figure 2.6) and in vitro (Figure 2.8), it is very interesting to figure out the transcriptional function of BLH6. To test this, the protoplast transfection system previously used to demonstrate the transcriptional repression function of OFP1 (Wang et al., 2007) was employed. In this transfection system, illustrated in Figure 3.7 A, there are four different effector plasmids. The first transactivator gene encodes a chimeric protein consisting of the LexA DNA-Binding domain (DBD) fused to the herpes simplex virus VP16 activation domain (LD-VP16), driven by the CaMV35S promoter. The second one, the control effector plasmid, contains a 35S promoter driving the Saccharomyces cerevisiae Gal4 DBD alone (GD). The third effector gene encodes a chimeric protein consisting of the Saccharomyces Gal4 DBD fused to the herpes simplex virus VP16 activation domain (GD-VP16), driven by the CaMV35S promoter. The fourth effector is a chimeric protein consisting Gal4 DBD fused to BLH6 (GD-AtBLH6). The GUS reporter gene driven by the 35S promoter with both LexA and Gal4 DNA binding sites [LexA(2x)-Gal4(2x):GUS reporter gene], and Gal4 DNA binding site alone [Gal4-GUS], were used as reporter. Cotransfection of reporter Gal4-GUS with effector GD-VP16 transactivator gene (Figure 3.7A) caused strong activation of the GUS reporter gene. Cotransfection of reporter Gal4-GUS with effector GD induced a very low level of GUS reporter gene expression. These two reactions were used as controls. GUS activity measured after cotransfection of Gal4-GUS with GD-AtBLH6 resulted in no activation of Gal4-GUS, since activity remained very low relative to the GD alone control (Figure 3.7 B). Cotransfection of the LD-VP16 transactivator gene and the effector gene encoding only the Gal4 DBD (GD) resulted in strong activation of GUS reporter gene. However, GUS enzyme activity after cotransfection of LD-VP16 with GD-AtBLH6 was lower relative to protoplasts transfected with the GD alone (Figure 3.7 C). The result indicates that BLH6 negatively regulates VP16 activated gene expression and thus functions as a transcriptional repressor.
Figure 3.7 Test of the transcriptional activity of BLH6
(A) Effectors and reporter constructs used in the transfection assays. (B) Transfection assay in which GD-AtBLH6 was co-transfected with the constitutively expressed reporter Gal4-GUS in the absence of transactivator. (C) AtBLH6 represses the expression of the reporter activated by a transactivator LD-VP16. Effector genes, transactivator and reporter genes were co-transfected into protoplasts derived from Arabidopsis rosette leaves. GUS activity was assayed after protoplasts had been incubated in darkness for 20-22 hr.

3.4 Discussion
In this Chapter, I examined the phenotypes of blh5 and blh6 knock-out mutants, with a focus on inflorescence stem anatomy and secondary wall formation. Based on the expression pattern of the corresponding gene (Ehlting et al., 2005), BLH5 was originally a strong candidate for interaction
with KNAT7. However, phenotypic analysis of the blh5 loss of function mutant and overexpression mutant failed to reveal any differences relative to wild-type plants. This is consistent with data obtained later, which showed that interaction between BLH5 and KNAT7 was undetectable in BiFC assays (Figure 2.6) and very weak in our yeast two hybrid assay (data not shown). Taken together these data suggest that BLH5 is not an interacting partner of KNAT7. Thus, while BLH5 is strongly upregulated during inflorescence stem development (Ehlting et al., 2005), its function remains to be determined. It is possible that functional redundancy between BLH5 and other members of BELL family, such as BLH1, exists, given their overlapping expression patterns and sequence similarities. This could be tested by generating a blh5 blh1 double mutant.

In terms of protein-protein interaction assays, data presented in Chapter 2 showed that BLH6 interacts with KNAT in vivo and in vitro. Initial phenotypic analysis of a blh6 loss-of-function mutant indicated that the mutant has somewhat thicker interfascicular fiber secondary cell walls, which if confirmed, would partially phenocopy knat7. However, the blh6 mutant did not display an irx phenotype, which is also a characteristic phenotype of knat7 loss-of-function mutants. This suggests that, as a KNAT7 interacting partner, BLH6 could be required for a subset of KNAT7 functions.

According to the phylogenetic trees described in Chapter 2 (Figures 2.1 A-C), BLH7 is a closely related paralog of BLH6. blh7 blh6 double mutants could be generated to test for functional redundancy between these genes, but the lack of BLH7-KNAT7 interaction suggests that BLH7 has a function at least partially distinct from that of BLH6. While I could not confirm the reported yeast two hybrid interactions of three other BLH proteins with KNAT7 (BLH5, BLH7 and ATH1) reported by Hackbusch et al. (2005) using the BiFC system (Figure 2.6), such potential interactions could be tested using other methods. If such interactions with KNAT7 were to occur, functional redundancy with BHL6 would be possible, perhaps explaining the apparent
mild phenotype of \textit{blh6} relative to \textit{knat7} mutants.

The data from the transcriptional activity assay (Figure 3.8) pointed out that BLH6 functions as a moderate transcriptional repressor in this assay system. The phenotype of the \textit{blh6} loss-of-function mutant which shows an apparent increase of the interfascicular cell wall thickness, similar to that seen for loss of KNAT7 function, which is known to be a transcriptional repressor (Li, 2008; Li et al., submitted). This preliminary evidence indicates that BLH6 may play a role in secondary cell wall formation in concert with KNAT7, consistent with the model we proposed in Figure 2.14. According to this model, BLH6 is a member of a BLH-KNAT7-OFP complex that functions to repress transcription. It will be interesting to use the same system to determine the transcriptional activity of BLH6-KNAT7 and BLH6-KNAT7-OFP complexes predicted to form when the proteins are co-expressed together in protoplasts.

Several aspects of the \textit{blh6} phenotype remain to be investigated, and it would be of interest to examine the phenotype of a \textit{knat7 blh6} double mutant. However, the results presented in this chapter, together with the protein-protein interaction data in Chapter 2, provide evidence in support of a role of BLH6 in the regulation of secondary cell wall formation through its interaction with KNAT7 as a part of a KNOX-B LH-OFP complex.
Chapter 4. Conclusion and future directions

Through use of reverse genetics and protein-protein interaction assays, I identified BLH6 from the six candidate BLH proteins as a BLH interacting partner of KNAT7. Furthermore, I have demonstrated that BLH6 is a transcriptional repressor and it is likely involved in secondary cell wall formation. However, much remains unknown regarding the existence of the BLH6-KNAT7-OFP complex and the biological function of this complex. In this chapter, I propose some research that could be pursued in the future.

4.1 BLH expression patterns

If BLH6 functions together with KNAT7 as part of a regulatory complex in vivo, it would be expected to have expression patterns that overlap with KNAT7. KNAT7 had previously been shown to be expressed in developing fibers and vascular bundles in inflorescence stems (Zhong et al., 2008). Furthermore, promKNAT7:GUS fusion expression is closely associated with the vascular system and interfascicular fibers in Arabidopsis seedlings, roots, and inflorescence stems (Li et al., submitted). In Chapter 2, I conducted an in silico analysis for expression patterns of BLH proteins. In order to provide better information on BLH6 expression patterns and to determine if they overlap with those of KNAT7, we would need to examine the cell-type expression pattern of a promBLH6:GUS fusion transformed into wild type Arabidopsis.

4.2 Functional analysis of the KNAT7-BLH6 complex

To study the biological function of KNAT7-BLH6 complex, an interesting experiment would be to generate a blh6 knat7 double mutant, and determine if the mutant has an additive phenotype, or one similar to knat7 or blh6. In terms of the phylogenetic analysis of BELL family, BLH7 is a close paralog of BLH6, suggesting that there might be functional redundancy between two genes. To test this, the blh7 loss-of-function mutants would have to be identified, the blh6 blh7 double mutant generated, and its phenotype compared to that of the single mutants. Considering that
4CL1: KNAT7 shows thinner interfascicular fiber cell walls than WT plants (Li, 2008), it would be worthwhile generating 4CL1: BLH6 overexpression lines, and compare the phenotype to that of 4CL1: KNAT7 lines. To further test whether BLH6 is required in the KNOX-BLH-OVATE complex, one could generate blh6 ofp4 double mutants and a blh6 knat7 ofp4 triple mutants by crossing loss-of-function mutants, then identify the homozygotes in the F2 generation by PCR genotyping. If the overexpression phenotype of KNAT7 in the stem requires these BLH proteins to cause decreased interfascicular fiber cell wall thickness, we might expect that loss of function of the corresponding BLH gene would suppress the phenotype. To test this possibility, we could generate the 4CL1:KNAT7 overexpression line in blh6 knockout background. Conversely, we could cross a 4CL1:BLH6 overexpression line to the knat7 knock out background to test whether the potential phenotype of BLH6 overexpression mutant depends on KNAT7 function. We also can cross 4CL1:BLH6 to 4CL1:KNAT7 overexpression lines to determine if the overexpression phenotypes are stronger. Based on the comparison of different mutant phenotypes, it should be possible to gain evidence about whether BLH6 and KNAT7 work in the same or different pathways. All the double and triple mutants planned are listed in Table 4.1 below.

### Table 4.1 Double and triple mutants to be generated

<table>
<thead>
<tr>
<th>Double Knock-out Mutant</th>
<th>Overexpression Mutant</th>
<th>Triple Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>blh6 blh7</td>
<td>4CL1:BLH6 knat7 KO mutant</td>
<td>blh6 knat7 ofp4 triple mutant</td>
</tr>
<tr>
<td>blh6 knat7</td>
<td>4CL1:KNAT7 blh6 KO mutant</td>
<td>blh6 blh7 knat7 triple mutant</td>
</tr>
<tr>
<td>blh6 ofp4</td>
<td>4CL1:BLH6 4CL1:KNAT7 mutant</td>
<td></td>
</tr>
</tbody>
</table>

As blh6 loss-of-function mutant exhibits possible altered S lignin amount in interfascicular fibers, it will be worth quantifying the total lignin content and amount of G and S subunits of the transgenic plants together with the control plants by chemical analysis. Since BLH6 may control other secondary cell wall properties, it would be worthwhile determining total lignin, cellulose,
and hemicellulose levels in the \textit{blh6} mutant, relative to wild type, and assay the cell wall phenotype of a second \textit{blh6} allele.

4.3 **Analysis of a KNOX-BLH-OVATE complex by protein-protein interaction assays**

The interaction between KNAT7 and OFP4 and OFP1 has been shown \textit{in vivo} and \textit{in vitro} (Li et al., submitted). In Chapter 2, I demonstrated the interaction between BLH6 and KNAT7 \textit{in vivo} and \textit{in vitro}, while BLH6 interaction with OFP4 was not detected by BiFC (data not shown) or yeast two-hybrid analysis (Figure 2.8). The question will be how to further test the protein-protein interactions within the KNOX-BLH-OVATE complex. One useful technique would be yeast three hybrid analysis to test whether KNAT7 could act as a scaffold, binding both BLH6 and OFP4 at the same time, if the predicted KNOX-BLH-OVATE complex containing these proteins exists.

Another way to test the interactions would be to use the protoplast transfection system described in Chapter 3 to determine the transcriptional activity of BLH6-KNAT7 and BLH6-KNAT7-OFPs complexes by cotransflecting 35S:HA-KNAT7 and 35S:HA- OFP4 with LexA(2x)-Gal4(2x):GUS reporter gene, the LD-VP16 effecter gene and the second effecter GD-AtBLH6 gene into protoplasts, and then measure GUS reporter expression levels. These experiments would show whether BLH6 and OFP4 work together to further enhance transcriptional repression by KNAT7 in a protein complex, or whether the proteins have antagonistic effects on transcriptional activity.

4.4 **KNAT7 target genes**

Microarray transcriptome profiling is a useful tool to detect gene expression change in the full-genome level, and I performed preliminary work using a DEX-induction system in which a 35S:KNAT7-GR construct was transformed into the \textit{knat7} mutant background. I showed that DEX induction of 35S:KNAT7-GR rescued the \textit{knat7} mutant phenotype. These experiments could
be continued to identify KNAT7 target genes. Candidate targets could be further investigated by RT-PCR expression profiling, conserved cis-acting regulatory elements identified, binding of recombinant KNAT7 to putative target promoters and cis-elements tested using EMSA, and reverse genetic analyses used to test the biological functions of the target genes. It would also be possible to introduce 35S:KNAT7-GR into ofp4 and blh6 mutant backgrounds, and determine differences in KNAT7-induced gene expression changes relative to WT. This approach could shed light on different functions of KNAT7-containing complexes.
References


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