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Thermocycle-regulated WALL REGULATOR INTERACTING bHLH Encodes a Protein That Interacts with Secondary-Cell-Wall-Associated Transcription Factors

Ian P. Whitney
University of Massachusetts Amherst

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A Thesis Presented

by

Ian P. Whitney

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Approved as to style and content by:

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Samuel P. Hazen, Chair

_____

Ludmila Tyler, Member

_____

Om Parkash Dhankher, Member

_____

Barbara A. Osborne, Program Head
Molecular and Cellular Biology

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ABSTRACT

THERMOCYCLE-REGULATED *WALL REGULATOR INTERACTING BHLH* ENCODES A PROTEIN THAT INTERACTS WITH SECONDARY-CELL-WALL-ASSOCIATED TRANSCRIPTION FACTORS

FEBRUARY 2015

IAN P. WHITNEY, B.S., UNIVERSITY OF MASSACHUSETTS AMHERST

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Samuel P. Hazen

Lignocellulosic biomass is one of the most abundant raw materials on earth that can be utilized to created carbon-neutral biofuels as a replacement for conventional fossil fuels. In order to create ideal energy crops, the regulation and deposition of cell wall polysaccharides must first be fully understood. Improved understanding of cell wall regulation will enable selection of traits that can optimize biofuel feedstocks. Herein, I utilize the grass model system *Brachypodium distachyon* in order to understand the transcriptional regulation of secondary cell wall deposition. Gene expression profiling was used to elucidate transcription factors that regulate secondary cell wall biosynthesis. Through this method, *WALL REGULATOR INTERACTING bHLH* (*WRIB*) was identified and its role as a secondary cell wall regulator was tested. Yeast-one- and yeast-two-hybrid assays showed that WRIB is capable of binding to promoters of secondary cell wall biosynthesis genes, as well as interacting with known secondary cell wall transcription factor proteins and also Phytochrome B. These results suggest that WRIB plays an important role in the secondary cell wall regulatory network and could perhaps be modulated by Phytochrome B. Discovery of this novel and interesting gene furthers the overall understanding of secondary cell wall development with the goal of improving our ability to engineer biofuel feedstocks.

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CHAPTER 1

INTRODUCTION

Fossil fuels power almost every aspect of the global economy; the waning supply has created demand for an alternative fuel source. One such alternative to petroleum is biofuel, which can be derived from the polysaccharides in plant cell walls. Lignocellulosic biomass is the most abundant source of polysaccharides on earth, which can be used for biofuel conversion. In addition to being cheap and plentiful, the conversion of lignocellulosic biomass into ethanol has a net-neutral or -negative impact on atmospheric CO_2 (Tilman et al., 2006). This net-negative carbon impact makes lignocellulosic biofuels a highly attractive option as they both alleviate our dependence on fossil fuels while remediating their harmful effects. As a result, there are concerted efforts to engineer high-yield bioenergy crops that can be efficiently converted into biofuels.

Two distinct layers of wall, the primary and secondary wall, can form outside the plasma membrane of plant cells. The primary wall is a relatively thin layer comprised mostly of cellulose and hemicelluloses and functions to determine cell shape. In contrast to the thin primary wall, the thick secondary wall makes up the majority of above-ground biomass. Plant secondary cell walls are deposited between the plasma membrane and the primary wall and consist primarily of three main components: cellulose, hemicelluloses, and lignin (Perez et al., 2002). These components are of particular interest to plant biofuel research as cellulose and hemicelluloses are polysaccharides that can be saccharified and fermented (Sorek et al., 2014). Deposition of secondary wall appears to be tightly regulated, as it is cell-type-specific and characteristic of certain developmental

stages (Matos et al., 2013). In addition to the sugar contained in these walls, lignin is also present in considerable quantity. Lignin is a polyphenolic compound that is highly recalcitrant to mechanical or enzymatic degradation. As a consequence, the bioconversion process requires harsh and expensive pretreatments in order to yield substantial biofuel (Huang et al., 2011). While lignin is essential to plant development due to its role in cell type specification and structural support, several studies have shown that a reduction in total lignin increases bioethanol yields for species such as maize (*Zea mays*), sorghum (*Sorghum bicolor*), and tall fescue (*Festuca arundinacea*) (Chen et al., 2003; Dien et al., 2009; Brenner et al., 2010). Factors such as polysaccharide content, biomass accumulation, and secondary cell wall composition also have a large impact on biofuel conversion, but are not fully understood. Increased understanding of secondary cell wall deposition should provide the ability to modify crops in order to make feedstocks that yield more ethanol with fewer pretreatments.

In the U.S.A, bioethanol is primarily derived from corn starch. Corn is a well-established and highly productive crop and as a result provides substantial biofuel feedstock. However, numerous problems accompany large-scale production of corn-based ethanol. Corn requires excessive inputs of nitrogen and fossil fuels for large-scale agronomic production (Barnett et al., 2010). Additionally, because corn is a staple food and feed crop, competition for land use and impacts on food prices negate many of its benefits as a potential energy crop. An ideal energy crop should be cost-effective, have high yields, and should not compete with food crops. In the search for an optimal energy crop, lignocellulosic biomass may be the most economical option. Grasses such as *Miscanthus giganteus* and switchgrass (*Panicum virgatum*) have many of the

characteristics of an ideal energy crop; large stature, the ability to grow on marginal lands, and low input requirements (Carroll and Somerville, 2009). However, these grasses are poorly suited to laboratory research due to their size, complex genetics, and long lifecycles. Given this, a model plant system should be used to gain understanding of larger energy crops that are not conducive to laboratory research.

The most studied and best characterized model plant is the eudicot *Arabidopsis thaliana*. In *A. thaliana*, the regulation of biomass accumulation and the biosynthesis of cell wall components is best understood, with many of the genes involved in these processes functionally characterized (Zhong et al., 2010; Wang et al 2012). This has led to a fundamental understanding of cell wall regulation in *A. thaliana* as a model for eudicots (Handakumbura and Hazen 2012; Fig. 1A). The current model in *A. thaliana* consists of a hierarchy of master regulators that control lesser regulators, which in turn act to alter biosynthetic gene expression. Central to all levels of this hierarchy are MYB transcription factors. MYB transcription factors are ubiquitous throughout plant species and have been shown to regulate a wide range of plant processes including cell fate determination, metabolism, development, and response to abiotic/biotic cues (Dubos et al., 2010). MYB functions are regulated in myriad ways: phosphorylation, microRNAs, and commonly through protein-protein interactions with bHLH transcription factors (Dubos et al., 2010). *At*MYB26 is a transcription factors that regulates expression of *SECONDARY-WALL-ASSOCIATED NAC-DOMAIN PROTEIN 1*(*AtSND1*), *NAC SECONDARY WALL THICKENING FACTOR 1/2* (*AtNST1*), and (*AtNST2*) in anthers, AtMYB26 is also crucial to proper pollen function (Yang et al., 2007). These three transcription factors subsequently play a role in the activation of *AtMYB46*/*83*, master

regulators of secondary cell wall biosynthetic genes, as well as a suite of other MYB transcription factors (Hussey et al., 2013). Seven MYB transcription factors are regulated by *AtMYB46/83*: *AtMYB4/32/54/58/63/75/85*, as well as *KNOTTED ARABIDOPSIS THALIANA* (*KNAT*) *KNOTTED1-like* 7 (*KNAT7*). Overexpression of *AtMYB46* resulted in an increase in secondary cell wall activity of lignin, cellulose, and hemicellulose biosynthetic genes, suggesting *At*MYB46 is a positive regulator (Zhong et al., 2007).

Unfortunately, *A. thaliana* is only distantly related to the potential energy crops that are monocots. Grasses differ from eudicots in a variety of developmental characteristics (Vogel et al., 2008; Handakumbura et al. 2012). Grasses and eudicots display distinct differences in vasculature patterning and growth. Eudicot vasculature is derived from a central cambium layer while grass xylem and phloem differentiate at intercalary meristems. Differences also exist in cell wall monomer composition between the two; in general, grasses have far less pectin and more lignin (Vogel et al., 2008). Given that there are fundamental differences between grasses and eudicots, the regulatory mechanisms of secondary cell walls in these two families are likely to have unique components. To this end, it is critical to understand secondary cell wall regulation specifically in grasses.

The model grass *Brachypodium distachyon* is employed to study the cell wall regulatory network in grasses. *B. distachyon* has many model system attributes: a sequenced genome, anatomical similarity to larger bioenergy grasses, and amenability to genetic manipulation (Brkljacic et al., 2011). What little is known of grass cell wall biosynthesis pathways has been compiled from a several species including corn, switchgrass, rice (*Oryza sativa*), wheat (*Triticum aestivum*), and sorghum (Lam et al.,

1992; Shen et al., 2013; Zhong et al., 2011; Yoshida et al., 2013). Functional characterization of genes from these plants has often been carried out in heterologous systems, limiting the amount of endogenous data. Given this lack of grass-specific knowledge, *B. distachyon* is an excellent model system in which to further develop our understanding of the grass cell wall biosynthetic regulatory network. Although *B. distachyon* is a relatively new model system, down-regulation of the lignin biosynthesis genes cinnamyl alcohol dehydrogenase (*BdCAD1*) and caffeic acid *O*-methyltransferase (*BdCOMT4*) has been shown to lead to greater ethanol conversion rates (Trabucco et al., 2013; Dalmais et al. 2013; d'Yvoire et al. 2012). The cellulose synthase genes *BdCESA4* and *BdCESA7* are involved in secondary cell wall biosynthesis in *B. distachyon* (Handakumbura et al., 2013). In addition to these biosynthetic genes, the transcription factor *Bd*MYB48 has been shown to interact with these biosynthetic genes and up-regulate secondary cell wall biosynthesis.

Transcription factors and their targets often exhibit similar expression patterns either across cell type or throughout the course of a day. Further investigation of expression patterns has led to the discovery of many novel secondary cell wall genes in a multitude of plant species (Brown et al., 2005; Persson et al., 2005; Mutwil et al., 2011). Secondary cell wall genes are highly expressed in secondary wall-rich tissues and cycle similarly throughout the day in other species, and this co-expression pattern appears to hold true in *B. distachyon*. Current research suggests that putative cell wall transcriptional regulators are similarly expressed with the characterized cellulose and lignin synthetic genes. Using this expression pattern as a guide, the candidate gene *WALL RELATED INTERACTING bHLH* (*WRIB*) was discovered and tested for a potential role in the

secondary cell wall regulatory network. In an effort to better understand the transcriptional regulation of cell wall biosynthesis in grasses, we sought to investigate the candidate gene *WRIB*. RNA time-course analysis revealed *WRIB* appears to be regulated by temperature; specifically *WRIB* is up-regulated by cold temperature. Secondary cell wall regulators as well as biosynthesis genes follow the same expression pattern which is anti-phasic to primary elongation. In addition to following this unique expression pattern, WRIB is of interest as it would be the only characterized bHLH protein within the secondary cell wall network. These factors made WRIB an alluring candidate. Herein, protein-protein and protein-DNA interactions are characterized in an effort to reveal the function of WRIB within secondary cell wall regulation. Utilizing yeast-one-hybrid tests, we assessed whether WRIB is functioning as a direct regulator of biosynthetic genes. We show that WRIB is capable of binding to the promoters of cellulose and lignin biosynthetic gene *BdCESA7* and *BdCAD1*. A yeast-two-hybrid approach was employed to elucidate whether WRIB may function in a protein complex or as a heterodimer. To this end it was observed that WRIB directly interacts with a suite of putative secondary cell wall transcription factors. Given these results, we conclude that WRIB is a novel protein in the secondary cell wall regulatory network of *B. distachyon* whose discovery will lead to a better understanding of the unique grass cell wall regulatory network.

CHAPTER 2

MATERIALS AND METHODS

2.1 RNA time-course analysis

RNA was extracted from seedlings under diurnal, thermocycling, and free-run conditions. Diurnal conditions consisted of 28°C, twelve-hour days with the lights on and 12°C, twelve-hour nights in darkness. Thermocycling conditions consisted of 28°C, twelve-hour days with lights on and 12°C, twelve-hour subjective nights with the lights on. Free-run conditions consisted of constant 28°C days with the lights on. Plant growth conditions, mRNA extraction, and cDNA synthesis were completed as outlined in (Matos et al., 2014). Gene expression was assessed by looking at relative expression patterns over all conditions. Genes in the RNA-timecourse with a detected expression level less than 30 were deemed unexpressed for the purposes of this analysis.

2.2 Phylogenetic analysis

WRIB protein sequence as well as the closest 23 *A. thaliana* and *B. distachyon* bHLH protein sequences were found using Phytozome v10 database (http://www.phytozome.net/) as well as NCBI BlastP database (https://blast.ncbi.nlm.nih.gov/). Full-length amino acid sequences were analyzed in MEGA5 to generate multiple-sequence alignments, as well as phylogenetic trees (Tamura et al., 2011) Phylogenetic trees were created by using the Maximum Likelihood method based on the Poisson correction model. 500 bootstrap permutations were taken to represent the evolutionary history of the proteins that were analyzed. Initial trees for the search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, analysis involved 24 amino

acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 71 positions in the final dataset.

2.3 Yeast one-hybrid assay

Full-length *WRIB* cDNA sequence was cloned into pDEST22 (GAL4-AD prey vector, Life Technologies, Carlsbad CA) using the Gateway cloning system. For each target gene, three overlapping promoter fragments were cloned into the pGLAC vector (*WRIB*::*LacZ*) using Gateway cloning. In this study, the promoter region was determined to be the nearest 1 kb upstream of the start codon. The WRIB protein was tested against twelve separate promoter fragments from five biosynthetic genes. Bait and prey constructs were transformed into yeast strain Ym4271 through consecutive lithium acetate (LiAC) yeast transformations as outlined in (Geitz et al., 2007). Yeast colonies were screened for *LacZ* expression by looking at the protein product of *LacZ*, β-galactosidase (β-Gal). Ortho-Nitrophenyl-β-galactoside (ONPG) was added to yeast cultures to assay for β-Gal activity. In the presence of β-Gal, ONPG is cleaved into the monosaccharide galactose and the yellow molecule ortho-nitrophenol which allows for a straightforward colorimetric assay.

2.4 Yeast two-hybrid assay

The plasmid pGBKT7 (GAL4-BD bait vector) and pGADT7 (GAL4-AD prey vector) were obtained as part of the Matchmaker Gold Yeast-two-hybrid system from Clontech (Clontech, Mountain View, CA). These vectors were modified with the addition of the Gateway cloning sites for ease of cloning. Full-length cDNA sequences for thirty putative secondary cell wall transcription factors were cloned into pGADT7 to be tested against WRIB protein. *WRIB* full-length cDNA sequence was similarly cloned into

pGBKT7 and tested for interactions. The bait vector (pGBKT7) conferred resistance to the antibiotic kanamycin and the ability to grow on media lacking tryptophan, while the prey vector (pGADT7) conferred ampicillin resistance and the ability to grow on media lacking leucine. In addition to the selective markers inherent in the vectors, the two *Saccharomyces cervisiae* strains also contained selectable markers. The bait yeast strain (Y2H gold) had selectable markers for adenine and histidine biosynthesis as well as resistance to the fungicide aureobasidin A. The prey yeast strain (Y187) contained selectable markers for α-galactosidase as well as β-gal. The bait and prey constructs were transformed into the appropriate yeast strains by utilizing highly efficient LiAC transformation (Geitz 2007). Interactions were determined on the highest stringency media, which lacked four essential amino acids (-His/-Leu/-Trp/-Ade) and also contained the harsh fungicide aureobasidin A (AbA). These plates also contained x-α-galactosidase, which allowed for blue/white screening.

2.5 Protein extraction

B. distachyon accession Bd21-3 plant tissue was used for protein extraction. Plants were grown in 20 hour, 28°C days with 4 hour, 12°C nights. Seeds were imbibed on moist paper towels for 7 days and planted in autoclaved potting mix (#2 Conrad Fafard Inc., Agawam, MA). Whole plant samples stage 47 on the *B. distachyon* BBCH-scale (Hong et al., 2011) were harvested during either the middle of the day or middle of the night period and immediately stored at -80°C. Whole plant tissue of individual plants was ground at -80°C using a ball mill until fully pulverized. Tissue was not allowed to thaw and was immediately treated with protein extraction buffer (50 mM Tris-HCL pH 7.5, 5 mM Ethylenediaminetetraacetic acid **(**EDTA), 5 mM ethylene glycol tetraacetic

acid (EGTA), 2 mM Dithiothreitol (DTT), 1 Roche complete protease inhibitor tablet (Roche, Basal, Switzerland), 10 μg/ml DNAse) at a buffer to sample ratio of 2:1. Samples were incubated on ice for 20 minutes and subsequently centrifuged at 16,000 *g* for 20 minutes at 4°C.

2.6 Isolation of heterologously expressed WRIB protein

Full-length *WRIB* cDNA sequence was cloned into vector pDEST15 using the Gateway cloning system. This vector allowed for the inducible expression of an N-terminal Glutathione *S*-transferase (GST)-tagged fusion protein, GST:WRIB, in *Escherichia coli* strain BL21a. A 250 mL culture of LB-Luria media was grown to OD260/280 of 0.3 and then induced over-night using a concentration of 4% arabinose. The cells induced to produce GST:WRIB were pelleted by centrifugation and then resuspended in 1x PBS. The cell suspension was then treated with lysozyme (10mg/ml 150 μL) for 30 minutes on ice and then sonicated three times for 30 seconds each. The lysate was then incubated with glutathione sepharose 4B (GE Healthcare, Piscataway, NJ) which bound to the GST-tagged proteins. Tagged proteins were washed three times with elution buffer (10 mM reduced glutathione, 50 mM Tris-HCL pH 8.0, 5% glycerol) to separate GST:WRIB from the agarose beads.

2.7 Antibody testing

Total plant protein extract, as well as GST:WRIB, was boiled 5 minutes at 98°C in SDS reducing buffer, (10% w/v sodium dodecyl sulfate (SDS), 10mM Beta-mercapto-ethanol, 20% v/v glycerol, 200 mM Tris-HCl, pH 6.8) prior to being resolved on a 10% polyacrylamide gel. Proteins that were resolved in the SDS gel were subsequently

transferred to a nitrocellulose membrane at 12V for 2 hours. Nitrocellulose membranes were next incubated in protein blocking buffer (Tris-Buffered saline solution (50 mM Tris-Cl, pH 7.5, 150 mM NaCl), 0.1% Tween20 with 5% w/v nonfat dry milk) for one hour. In order to carry about these experiments under native conditions, an anti-WRIB antibody was raised in rabbit against the specific peptide sequence (CYQKLSQQQSQPGNSKQ) which is located from amino acids 394-409 of the WRIB protein (Yenzyme, San Francisco, CA). Membranes were subsequently incubated with a 1:2,500 dilution of the anti-WRIB primary antibody overnight at 4°C. After being washed five times in 1X TBST, the membrane was incubated for 1 hour in the anti-rabbit radish peroxidase secondary antibody (GE Healthcare) at a buffer to antibody ratio of 1:5000 in TBST. Blots were then washed five times in TBST to remove residual, unbound antibody. Blots were next incubated for 4 minutes in the chemiluminescent solution (100 mM Tris-HCl, pH 8.5, 2% H_2O_2, 85 mM p-coumaric acid, 0.2 mM luminol) to allow detection using a G-box iChemi gel documentation system (Syngene, Cambridge, UK) for visualization at 428 nm.

2.8 Gene numbers

AtMYB26 (At3g13890), AtNST1 (At2g46770), AtNST2 (At3g61910), AtSND1 (At1g32770), AtMYB46 (At5g12870), AtMYB83 (At3g08500), AtMYB4 (At4g38620), AtMYB32 (At4g34990), AtMYB54 (At1g73410), AtMYB58 (At1g16490), AtMYB63 (At1g79180), AtMYB75 (At1g56650), AtMYB85 (At4g22680), AtKNAT7 (At1g62990), BdCAD1 (Bradi3g17920), BdCESA4 (Bradi4g28350), BdCESA7 (Bradi2g30540), BdCESA8 (Bradi2g49912), BdCOMT4 (Bradi3g16530), BdMYB48 (Bradi2g47590), BdWRIB (Bradi3g33170), BdGNRF (Bradi2g46197), AtPIF4 (At2g43010), AtPIF5

(At3g59060), AtPIF7 (At5g61270), AtPIL1 (At2g46970), AtPIL2 (At3g62090), AtPIL5 (At2g20180), AtHFR1 (At1g02340), AtSPT (At4g36930), AtALC (At5g67110), AtUNE10 (At4g00050), AtPIF3 (At1g09530), AtMYB42 (At4g12350), AtPHV (At1g30490), AtPHB (At2g34710), BdPHYA (Bradi1g10520), BdPHYB (Bradi1g64360), BdPHYC (Bradi1g08400), AtPHYB (At2g18790), AtOFP4 (At1g06920), AtARP6 (At3g33520), AtELF3 (At2g25930), AtELF4 (At2g40080), AtLUX (At3g46640) were mentioned in this research, see Table 2 for yeast-two-hybrid gene numbers.

CHAPTER 3

RESULTS

3.1 Discovery of *WALL RELATED INTERACTING bHLH*

To understand gene expression patterns in the *B. distachyon* transcriptome under different photo- and thermocycle conditions, mRNA time-course data were analyzed. Plants were sampled every 3.5 hours over the course of two days under diurnal, thermocycling, and free-run conditions. RNA-sequencing was used in order to uncover genes that are co-regulated with secondary cell wall genes, the transcriptome was searched for genes with similar expression patterns ($r > 0.75$) to the characterized cell wall genes *BdCAD1, BdCOMT4,* and *BdCESA4/7/8*. Only two transcription factors met this criterion for four of the five aforementioned genes. The first gene, *GNRF*, is currently being functionally characterized in the Hazen Lab. *GNRF*, an ortholog to the transcription factor *AtSND2*, plays a role in the regulation of secondary cell wall deposition, vascular patterning, and flowering time. The second gene with a similar expression pattern to the cell-wall-related genes was *WRIB* (Fig. 2). Expression patterns for normal diurnal conditions show that *WRIB* and the cell wall biosynthesis genes are expressed highly at night (Fig. 2). Both *WRIB* and the genes for these biosynthetic enzymes had a similar expression pattern under thermocycling and diurnal conditions, with peak expression during subjective night. In contrast, under free-run conditions, the secondary cell wall genes and *WRIB* did not follow any distinct cyclical pattern.

3.2 Analysis of WRIB protein sequence

WRIB is a type VII bHLH transcription factor 452 amino acids in length. This protein contains a well-conserved Helix-Loop-Helix (HLH) domain spanning the 172

amino acids from 208-390 (Figure 3A). In addition to this, WRIB contains an N-terminal Active Phytochrome Binding (APB) motif spanning amino acids 10-24, a motif that is well conserved throughout the Phytochome Interacting Factors (PIFs) (Figure 3B). WRIB protein also contains a nuclear localization signal located from amino acid 225-230.

Preliminary phylogenetic analysis of WRIB revealed the closest *A. thaliana* orthologs include AtPIF4, AtPIF5, AtPIF7, as well as AtPIL1, AtPIL2, and AtPIL5 (Figure 4). In addition to these well-characterized phytochrome-interacting bHLH's, WRIB clustered with LONG HYPOCOTYL IN FAR RED (AtHFR1), SPATULA (AtSPT), and ALCATRAZ (AtALC). Further analysis of type VII bHLH proteins shows that WRIB forms a sub-clade with *A. thaliana* UNFERTILIZED EMBRYO SAC10 (AtUNE10) as well as AtPIF7. AtPIF7 works in conjunction with AtPIF3 and AtPIF4 to control phytochrome B (AtPHYB) protein and transcript levels in response to red light (Leivar et al., 2008). AtUNE10 is a bHLH protein that plays an unclear role in embryo fertilization (Feller et al., 2011).

3.3 WRIB Protein-DNA interacting partners

Given that type VII bHLH proteins have been shown to bind to DNA and modulate gene expression (Heim et al., 2003), a yeast one-hybrid screen was performed with promoter fragments of secondary cell wall biosynthetic genes *BdCESA4/7/8*, *BdCAD1*, and *BdCOMT4* (Fig 5A, Table 1). Full-length WRIB protein interacted with the distal promoter fragment of the cellulose synthase gene *BdCESA7*, located from 1000-500 base pairs upstream of the start codon (Table 1). WRIB also interacted with the regulatory region of the lignin biosynthesis gene *BdCAD1*, where WRIB bound to the

proximal promoter fragment, which consisted of the closest 500 base pairs upstream of the start codon. Positive interacting promoter fragments were further analyzed in order to determine a possible binding motif for WRIB. MEME (multiple expectation maximization for motif elicitation) was utilized in order to illustrate possible binding motifs for WRIB (Bailey and Elkan 1994). The two highest likelihood binding motifs for WRIB are GG(gg/cc)GGGCC and TCACCT(c/g)CTCC (Fig 5B).

3.4 WRIB Protein-protein interactions

bHLH proteins have been shown to function as homo- or heterodimers in order to modulate gene expression (Heim et al., 2003). Therefore, a yeast-two-hybrid screen was performed in order to test for protein-protein interactions of WRIB with itself and WRIB with known secondary-cell-wall-related transcription factors. WRIB did not appear to homodimerized and of the thirty putative secondary wall transcription factors screened, twelve interactions with WRIB were identified (Table 2). Six MYB transcription factors, BdMYB18/31/36/44/48/69, were shown to interact with WRIB (Figure 6). Amongst these MYBs, *BdMYB48* has been well characterized in the Hazen Lab as influencing distinct secondary cell wall phenotypes in overexpressor and dominant repressor lines. Mutant plants overexpressing *BdMYB48* showed slightly increased secondary cell wall development, while lines expressing *BdMYB48* fused with a dominant repressor motif appeared to have thinner walls and were more digestible in a microbial conversion assay (Unpublished data, Handakumbura 2014). *BdMYB18* is a close homolog to *AtMYB50* and *AtMYB61*, which have been shown to control stomatal aperture (Liang et al., 2005). *BdMYB69* is a close homolog to *AtMYB42* and *AtMYB85*, which display altered expression when master regulator SND1 is modulated (Zhong et al., 2008).

In addition to the six MYB transcription factors, WRIB also interacted with BdNAC28, BdSWN6, BdHB7, BdbZIP57, BdBZIP61, KNOB1, and BdMADS29. BdSWN6 is a close homolog to VASCULAR RELATED NAC DOMAIN PROTEIN 7 (AtVND7), which plays a role in xylem development in stem tissue (Yamagachi et al., 2008). BdHB7 is a close homolog of PHAVOLUTA and PHABULOSA (PHV, PHB), which are involved in patterning of the lateral organs from the apical meristem (Emery et al., 2003).

Given the cold-regulated expression pattern that was observed of WRIB and other secondary cell wall genes, the expression patterns of genes that encode interacting proteins were tested. *BdMYB31*/*36*/*48*/*69* and *BdNAC28* all displayed a similar regulation pattern to *WRIB*: upregulation by cold ambient temperature (Fig 7). RNA-sequencing revealed that these genes displayed peak levels just before dawn and their expression appeared to be quickly downregulated in response to the warm ambient temperatures at dawn. In contrast, there was no discernable pattern in *BdMYB44* and *BdHB7* expression under any of the tested conditions (Fig 7). Unfortunately, *BdMYB18*, *BdSWN6*, *BdBZIP57/61*, and *BdMADS29* were not measured at a sufficient level to detect expression patterns.

WRIB shares homology with PIF and PIL proteins; of these, WRIB is most similar to AtPIF7. Considering that AtPIF7 protein interacts with AtPHYB, protein-protein interactions were tested between WRIB and BdPHYA/B/C in order to elucidate whether WRIB may function similarly. It was observed that WRIB physically associates with BdPHYB in yeast-two-hybrid testing (Fig 8). In *A. thaliana*, phytochromes play an integral role in regulating a wide range of processes including flowering, auxin

suppression, and organ development (Reddy et al., 2014; Casson et al., 2014). Although the WRIB protein-DNA and protein-protein interactions shown in yeast are both novel and relevant to secondary cell wall regulation, it is necessary to confirm these interactions *in planta* to further understand the function of WRIB.

3.5 *In planta* detection of WRIB protein

Observations of WRIB interacting with various putative secondary cell wall transcription factors in yeast warranted further investigation of how WRIB might function *in planta*. In order to do this, a native antibody was raised against WRIB protein. A 17-amino-acid epitope (CYQKLSQQQSQPGNSKQ) was chosen from the C-terminus of WRIB protein and used to raise polyclonal antibodies in rabbit (Fig 3A). This antibody was first validated by detecting recombinant WRIB protein. A GST:WRIB fusion protein was expressed and purified using the pDEST15 vector in BL21-AI *E. coli* cells. Western blot analysis showed that anti-WRIB was able to bind a purified protein of the expected size for WRIB (Fig 9). Specificity of anti-WRIB was then tested in whole-plant protein extracts. Whole plants were harvested during both day and night just prior to inflorescence emergence. Total protein extracts were subsequently probed with anti-WRIB. Western blot analysis of these whole-plant extracts showed that the WRIB antibody bound a plant protein of the expected size of 44 KDa (Fig 9).

CHAPTER 4

DISCUSSION

Basic helix-loop-helix transcription factors are a large family that has been well characterized in many species. BHLHs can function as homo- or heterodimers in order to bind DNA and modulate transcriptional activity (Quong et al., 1993: Littlewood and Evans 1998). Specifically, *WRIB* is part of the Type VII bHLH sub-family that includes PIF and PIL proteins, which integrate environmental, circadian, and hormonal inputs in order to regulate cell elongation and flowering (Braidwood et al., 2014; Heim et al., 2003). In *A. thaliana*, PIFs integrate light signaling largely through their interactions with PHYB. PIFs are activators of hypocotyl elongation, whereas PHYB negatively regulates this process by targeting PIFs for proteolysis (Al-Sady et al., 2006). This creates a regulatory pathway in which light signaling directly influences elongation.

Interestingly, hypocotyl elongation appears to be up-regulated by high temperature as well as light. Mutants in which *PIF4* is disrupted do not exhibit elongated hypocotyls in response to higher ambient temperatures; it has been shown that this phenotype is a result of a unique mechanism, not the canonical interactions with DELLA proteins or phytochromes (Koini et al., 2009). These data suggest that PIF proteins may also be involved in temperature perception. Although it is largely unknown how temperature sensing occurs in plants, recent findings have shown that AtARP6 functions as a part of the SWR1 complex which inserts H2A.Z nucleosomes in a temperature-dependent manner (Kumar and Wigge 2010). Similarly to *pif4* mutants, *arp6* mutants fail to integrate ambient temperature in order to adjust elongation rates compared with wild-type (Kumar and Wigge 2010).

The role that PIF proteins play in temperature sensing is also closely tied with the role they play in circadian clock regulation. In *A. thaliana*, the circadian clock regulators AtELF3/4 and AtLUX form what is known as the evening complex (EC). These proteins function together to maintain circadian-regulated gene expression and rhythmic hypocotyl elongation, as well as flowering (Nusinow et al., 2012). Further investigation has shown that AtPIF4/5 protein levels are tied to the EC and are regulated by recruitment by AtLUX. Functional EC as well as AtPIF4/5 are necessary for circadian-regulated hypocotyl regulation. Given this, the PIFs and close homologs could function as master regulators capable of integrating a suite of inputs in order to modify downstream gene expression.

In addition to the well-characterized PIF/PIL proteins, maize bHLH protein B forms an active transcriptional complex with maize MYB protein C1. This interaction is necessary for proper anthocyanin production in maize (Goff, Cone, and Chandler 1992). This is of interest given that anthocyanins are made through the same phenylpropanoid pathway which produces the monomeric forms of lignin (Fraser and Chapple 2011). BHLH proteins also function as heteromultimers in order to modulate transcription, such as the bHLH/MYB/WD40 complex that has been shown to regulate a wide range of biological functions in *A. thaliana*, petunia, and maize (Ramsay et al., 2005; Albert et al., 2009).

Genes encoding secondary cell wall transcription factors, as well as biosynthetic genes, display a clear peak in expression during cold subjective nights regardless of light input (Fig. 2). In contrast, *B. distachyon* leaf tissue has been shown to elongate in response to high temperatures irrespective of other conditions (Matos et al. 2014). While

the mechanism by which plants perceive temperature is unclear, it is apparent that plant functions are controlled by ambient temperature. AtPHYB has been shown to repress flowering when plants are grown at 23°C, but this repressive effect is lost at 16°C (Halliday 2003). *WRIB* and other secondary-cell-wall-related genes may be upregulated during cold periods as part of a temperature-regulated mechanism in which cells are expanding during the warm day periods and being reinforced with secondary cell wall polymers during cold night periods.

Yeast-one-hybrid screens uncovered that WRIB protein can associate with the promoter regions of the lignin biosynthetic gene *BdCAD1* and the cellulose biosynthetic gene *BdCESA7* (Fig 5). Interestingly, WRIB only associated with a single promoter fragment of each of these genes and did not associate with any of the other biosynthetic genes, although WRIB does appear to form many protein-protein interactions. This result could be due to the fact that WRIB is acting as an enhancing factor, similar to *A. thaliana* OVATE FAMILY PROTEIN4 (OFP4). AtOFP4 physically associates with KNAT7 and leads to enhanced repression on downstream targets (Li et al. 2011). WRIB could be associated with other proteins in order to modulate the efficacy of other secondary cell wall transcription factors like the MYBs that bind directly to lignin and cellulose biosynthetic genes.

Transcription factors often regulate gene expression through the formation of protein-protein complexes. These complexes have been shown to regulate gene expression as well as protein abundance in a wide range of organisms. Yeast-two-hybrid assays showed that WRIB interacted with a wide range of secondary cell wall transcription factors (Fig 6). For example, WRIB interacts with BdMYB69, a close

homolog of AtMYB42/85, which has been shown to be involved in secondary cell wall regulation (Zhong et al. 2008). In addition to this, WRIB interacts with BdMYB48, which has been functionally characterized and also plays a role in secondary cell wall regulation. In addition, WRIB physically associates with BdHB7 and BdSWN6, whose homologs are involved in stem development, organ organization, and secondary cell wall development. Of the interactions observed, there is a precedent for WRIB/MYB interactions. The importance of protein-protein interactions between bHLH and MYB transcription factors has been demonstrated in maize, *A. thaliana*, rice, and petunia (Grotewold et al., 2000; Qi et al., 2011; Zhang et al., 2009; Quattrocchio et al., 1998). Of particular interest are the bHLH/MYB interactions that alter the deposition of anthocyanins. Anthocyanins are created in the phenylpropanoid pathway, the same pathway in which lignin monomers are synthesized. Given this, it is feasible that WRIB/MYB interactions could be functioning through a similar mechanism to control the deposition of lignin in the secondary cell wall.

In addition to interacting with putative secondary cell wall transcription factors, WRIB also associated with BdPHYB. This interaction further validates *WRIB* as an *AtPIF7* homolog in *B. distachyon*. However, the fundamental differences between grasses and eudicots could dictate different functions for the two homologous proteins. Given the ubiquitous involvement of phytochromes in plant development, there are many processes that WRIB could be altering by physically associating with BdPHYB. Most interestingly, in light of the connection between the anthocyanin and lignin synthesis pathways, phytochromes have been shown to regulate nitrogen, phosphate, and anthocyanin accumulation in *A. thaliana* shoots (Oh 2014).

In summary, WRIB has been shown to form protein-protein interactions with secondary cell wall transcription factors and is also capable of binding the promoter regions of secondary cell wall lignin genes. *WRIB*, as well as many of its interacting partners, follows a temperature-driven expression pattern which is consistent with the expression of the secondary cell wall genes that have been characterized in *B. distachyon*. WRIB shares homology with the regulatory PIF transcription factors of *A. thaliana*, which integrate light, circadian rhythms, and temperature in order to regulate a wide range of plant functions. While WRIB shares many characteristics of PIFs, the grass system has many distinct differences from the eudicot model *A. thaliana*. As a result, WRIB could have substantially different downstream targets. While *A. thaliana* PIFs appear to regulate hypocotyl elongation with help from the circadian clock, *B. distachyon* hypocotyl elongation appears to occur devoid of circadian inputs (Matos et al., 2014). WRIB may function in response to temperature and light through interactions with BdPHYB in order to regulate secondary cell wall deposition by both binding directly to secondary cell wall biosynthesis genes and forming complexes with putative secondary cell wall transcription factors.

In order to understand this system fully, *in planta* assays must be performed to observe native WRIB behavior. Antibodies were shown to bind specifically to WRIB in *B. distachyon* plant extracts (Fig 9). This can function as the proof of concept for a wide range of interesting experiments. Utilizing anti-WRIB antibody, a sequential immuno-precipitation can be done with BdMYB48 to confirm the protein-protein interactions seen in the yeast-two-hybrid assay. In addition, co-immunoprecipitation/mass spectrometry experiments using anti-WRIB antibody could identify known and novel interacting

partners. Lastly, chromatin immunoprecipitation could be performed to validate the yeast-one-hybrid assays, as well as to determine additional DNA targets of WRIB. Although a wealth of knowledge would be gained from these experiments, functional characterization is still necessary to understand WRIB effects on a large scale.

Phenotypic characterization of *wrib* and *myb* mutant plants will yield valuable information regarding the effects of knocking out and overexpressing *WRIB in planta*. Currently, overexpression and *wrib* null lines are being created and should prove to be a valuable research tool. Given the expected role of *WRIB* in secondary cell wall biosynthesis, we anticipate phenotypes related to either a deficiency or an overabundance of cell wall polymers. This can be assayed through the use of simple histological analyses for lignin and cellulose. If *WRIB* functions as an activator of wall biosynthesis, I expect overexpression plants to display greater lignin and cellulose content; null lines are expected to exhibit decreased content. In contrast, if *WRIB* is a repressor of secondary cell wall biosynthesis, I expect overexpression lines to exhibit decreased lignin and cellulose, while null lines would show the inverse. The interplay between WRIB, Phytochrome B, and cell wall regulators can also be tested through the use of null mutants for these genes. Further investigation using *PhyB* null plants can help to understand how PhyB may be regulating WRIB protein levels. PhyB null plants can also be utilized to see if PhyB is regulating cell wall development. Lastly, temperature range experiments can be done to test for temperature specific functions of WRIB to see if WRIB is activating or repressing the cell wall regulation response to external cues. Functional characterization of WRIB would add a novel member to the secondary cell

wall biosynthesis transcriptional network in grasses, enabling further research in this field.

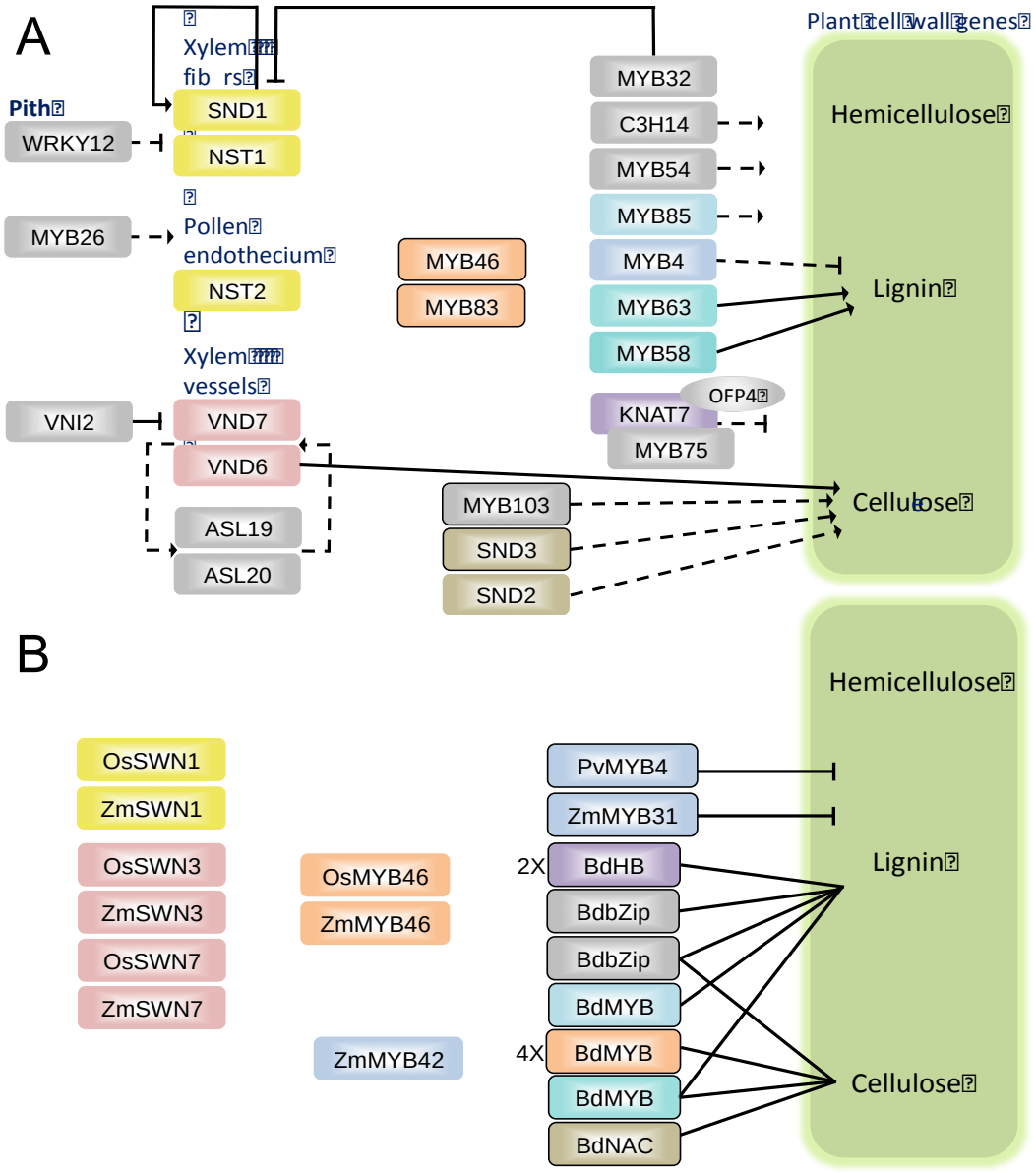

Figure 1. Secondary cell wall biosynthesis regulatory networks of *Arabidopsis thaliana* (A) and grasses (B). Rectangles represent transcription factors. The oval indicates an interacting protein. Solid arrows and bordered rectangles signify evidence for direct interactions. Dashed arrows indicate no evidence for direct interaction. T-bars denote a negative interaction and arrows denote a positive interaction. Lines connecting to the large green rectangles signify interactions with characterized cell wall biosynthesis genes. Orthology between *A. thaliana* and grasses is denoted by color. (Handakumbura and Hazen, 2012)

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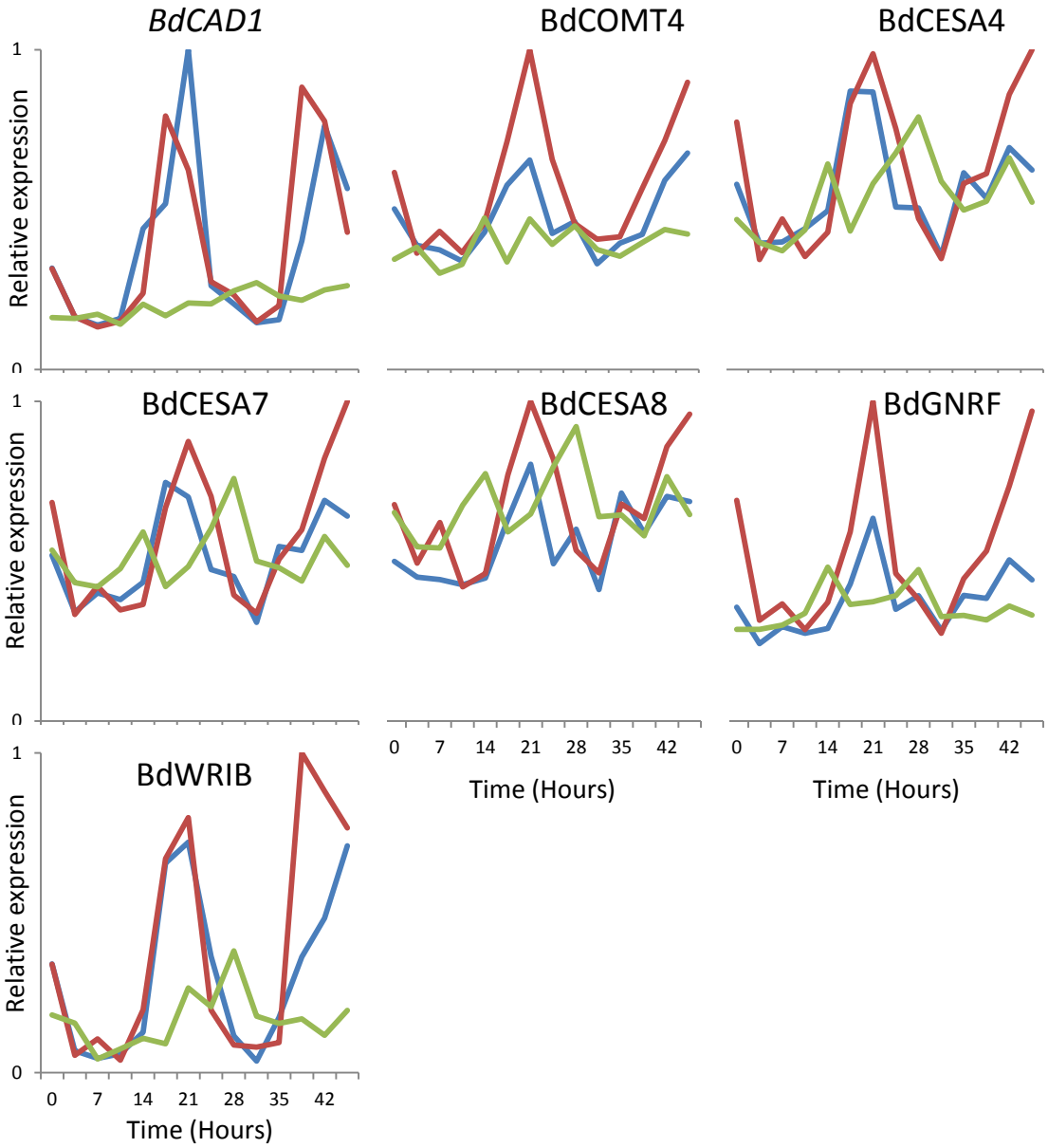

Figure 2. Gene expression analysis of putative secondary cell wall biosynthesis genes. RNA-time-course data of leaf tissue grown under diurnal (blue), thermo-cycling (red), and free-run conditions (green). Relative expression patterns of known cellulose synthase (*BdCESA4/7/8*), lignin synthesis (*BdCAD1/BdCOMT4*), secondary cell wall transcription factor gene *GNRF*, as well as *WRIB* are shown.

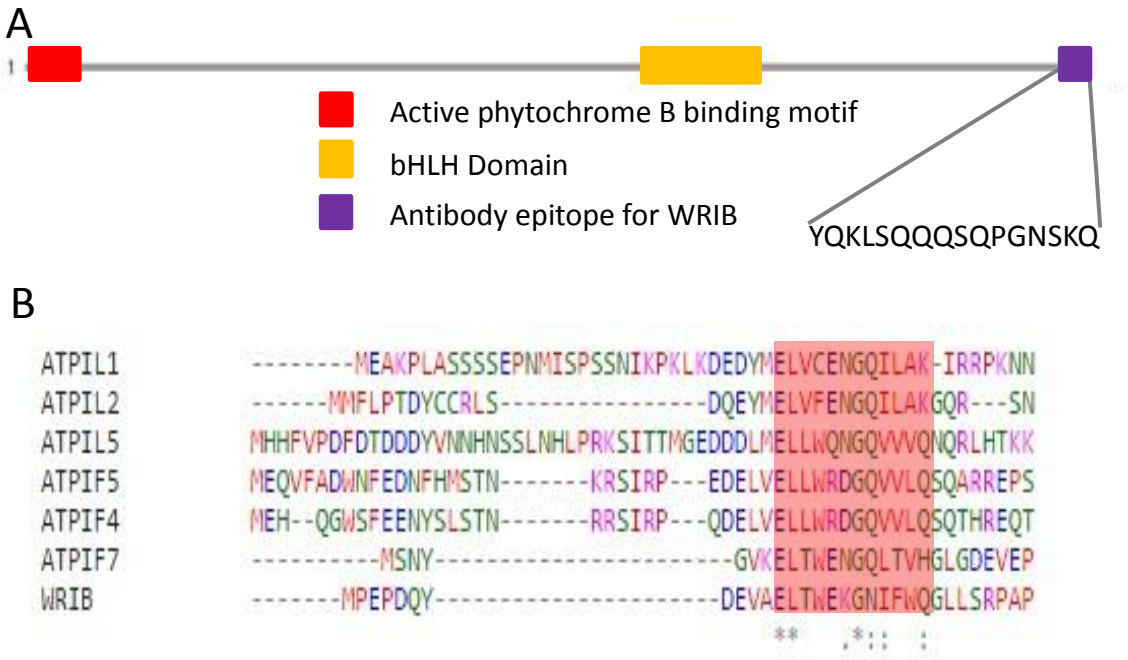

Figure 3. Analysis of WRIB protein domains. A.) Full-length WRIB protein with to-scale depictions of motifs. **B.)** Partial amino acid alignment from amino acids 1-31 of WRIB and six PIF/PIL proteins. The red highlighted sequences indicate the conserved Active Phytochrome B Binding Motif.

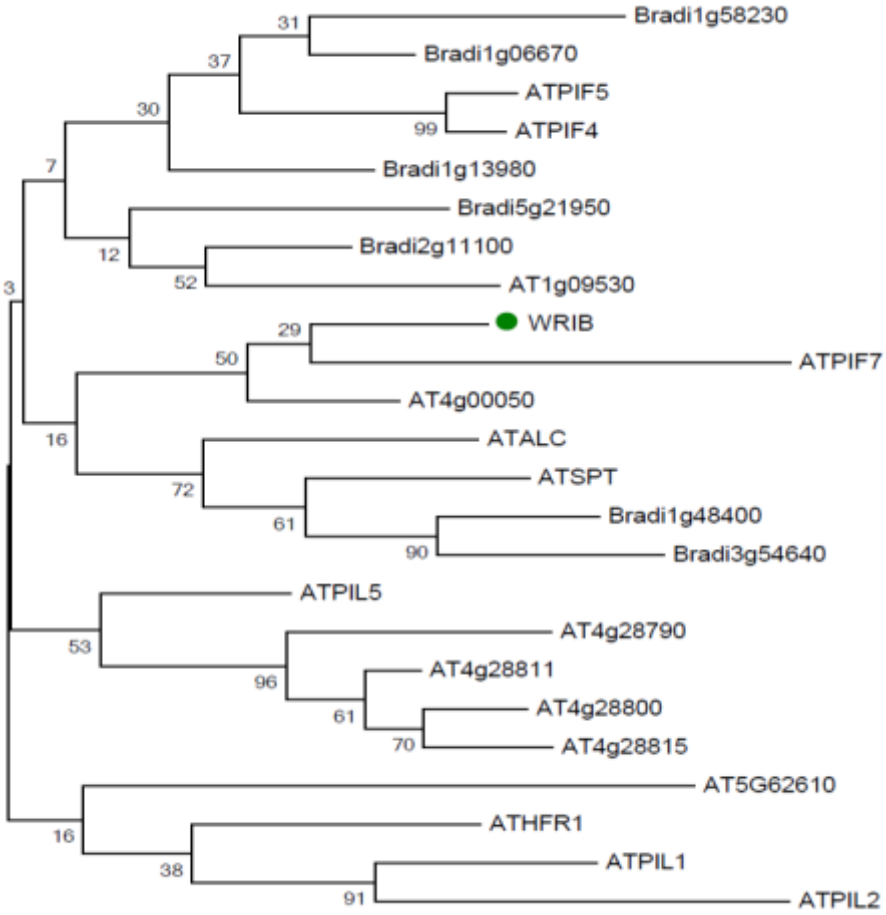

Figure 4. Molecular phylogenetic analysis of WRIB and the closest *Arabidopsis thaliana* and *Brachypodium distachyon* bHLH proteins by maximum likelihood method. Phylogenetic trees were created by using the Maximum Likelihood method based on the Poisson correction model. 500 bootstrap permutations were taken to represent the evolutionary history of the proteins that were analyzed. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, analysis involved 24 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 71 positions in the final dataset.

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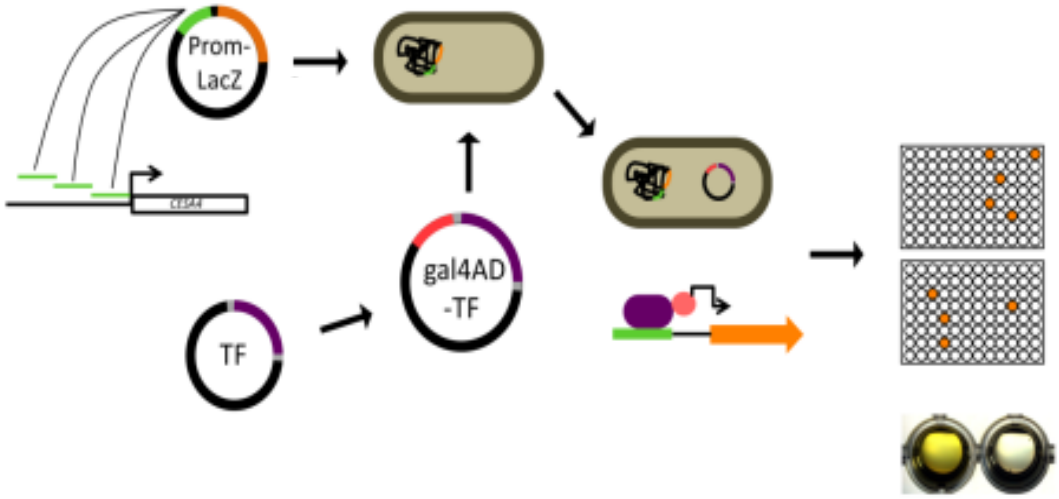
A



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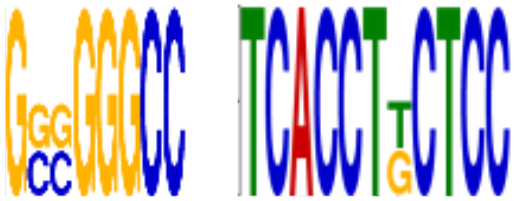

Figure 5. Yeast-one hybrid screen for *WRIB* DNA binding. A.) yeast-one hybrid workflow. Three overlapping promoter fragments of *BdCAD1, BdCOMT4, BdCESA4 BdCESA7,* and *BdCESA8* were tested in a LacZ reporter construct. These constructs were transformed into yeast together with a GAL4-activation domain (GAL4-AD)-transcription factor (TF) construct. In this study GAL4AD-WRIB construct was used. Positive interactions were screened for LacZ expression using β-galactosidase which resulted in a yellow color in the presence of LacZ expression products. **B.)** Common DNA binding motifs between positive interacting promoter fragments *BdCESA7* and *BdCAD1.*

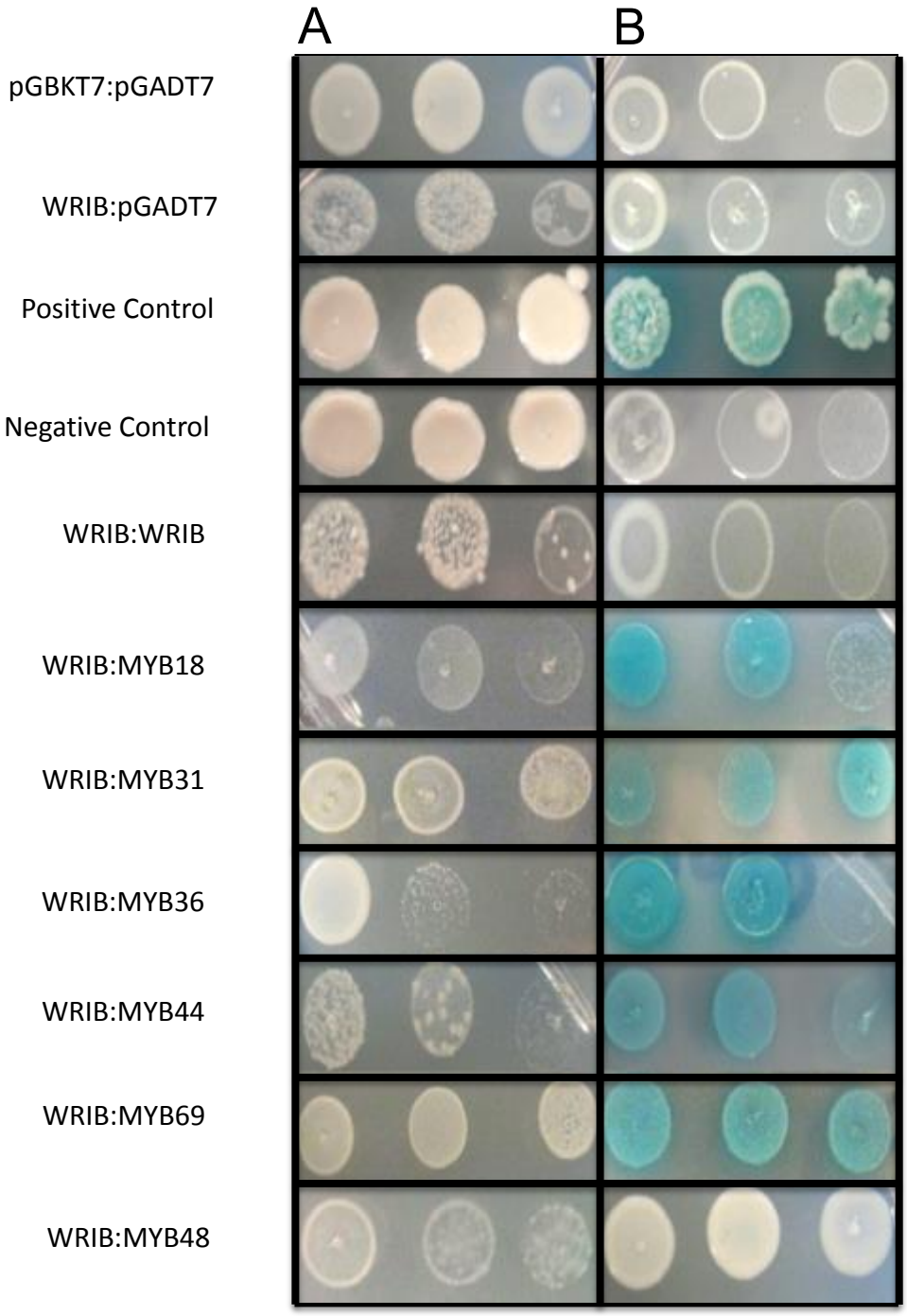

Figure 6. **Yeast-two hybrid testing between WRIB and putative secondary cell wall transcription factors.** *WRIB* was expressed in pGBKT7 (Gal4BD) and transformed into the Y2HGold yeast strain; preys were expressed in pGADT7 (Gal4AD) and transformed into the Y187 strain. *Bait*- and *prey*-transformed yeast were mated and the resulting diploids grown on (A) SD-Leu/-Trp medium, selecting for the presence of both plasmids and also on (B) SD-Leu/-Trp/-His/-Ade/X-α-gal/AbA medium, where only the diploids presenting protein interactions are capable of growth. 20-fold serial dilutions were performed prior to plating.

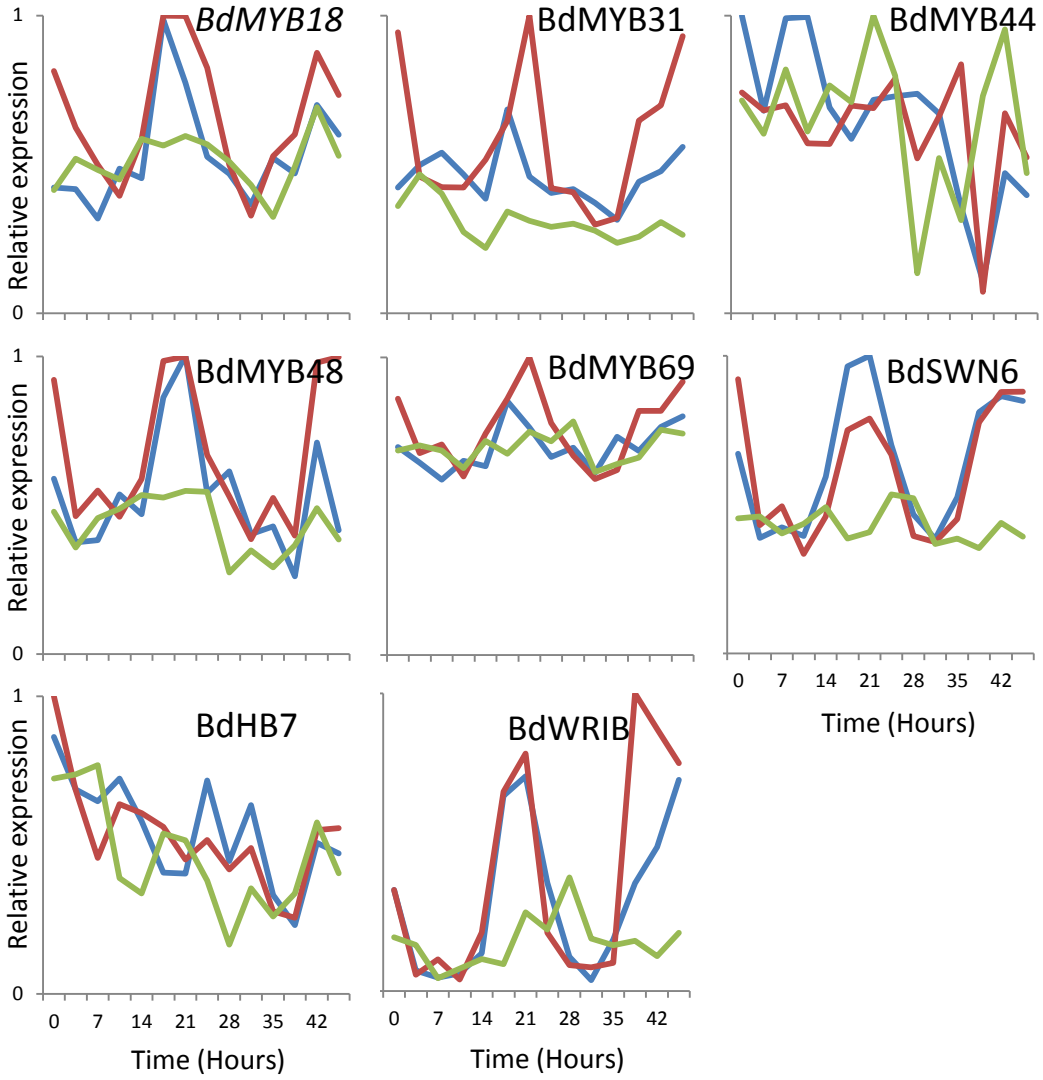

Figure 7. Gene expression analysis of WRIB-interacting transcription factors. RNA-time-course data of leaf tissue grown under diurnal (blue), thermocycling (red), and free-run conditions (green). Relative expression patterns of *BdMYB31/36/44/48/69, SWN6, HB7,* and *WRIB* are shown.

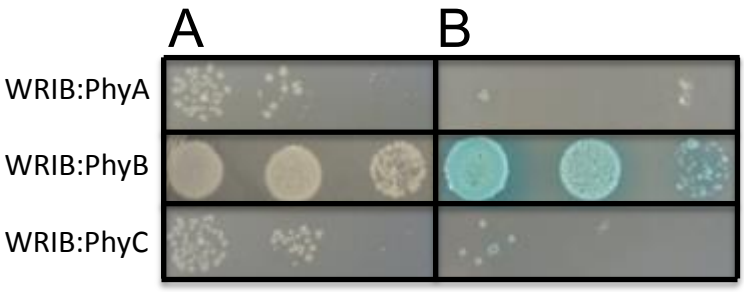

Figure 8. **Yeast-two hybrid testing between WRIB and Phytochrome A/B/C.** *WRIB* was expressed in pGBKT7 (Gal4BD) and transformed into the Y2HGold yeast strain; preys were expressed in pGADT7 (Gal4AD) and transformed into the Y187 strain. (A) *Bait*- and *prey*- were mated and the resulting diploids grown on SD-Leu/-Trp medium, selecting for the presence of both plasmids and also on (B) SD-Leu/-Trp/-His/-Ade/X-α-gal/AbA medium, where only the diploids presenting protein interactions are capable of growth. 20-fold serial dilutions were performed prior to colony plating.

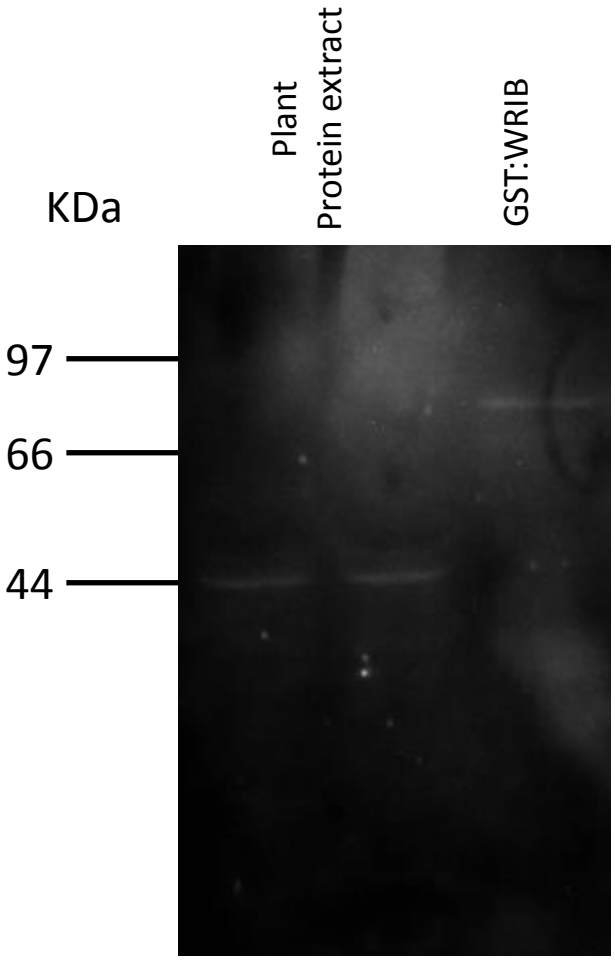

Figure 9. Antibody detection of WRIB protein in plant protein extracts. During the day (Lane 1) and night (Lane 2) as well as heterologously expressed GST:WRIB protein (Lane 3). 80 µg of the plant total protein extract was used in lanes 1 and 2 while 20 µg of the WRIB:GST protein was used in lane 3.

Table 1. Summary of protein-DNA interactions between WRIB and the promoter fragments of putative secondary cell wall biosynthesis genes

Promoter Fragment	Gene ID	Interaction
BdCADp-1	Bradi3g06480	Negative
BdCADp-2	Bradi3g06480	Negative
BdCADp-3	Bradi3g06480	Positive
BdCOMTp-1	Bradi3g16530	Negative
BdCOMTp-2	Bradi3g16530	Negative
BdCOMTp-3	Bradi3g16530	Negative
BdCESA4p-1	Bradi3g28350	Negative
BdCESA4p-2	Bradi3g28350	Negative
BdCESA4p-3	Bradi3g28350	Negative
BdCESA7p-1	Bradi4g30540	Positive
BdCESA7p-2	Bradi4g30540	Negative
BdCESA8p-1	Bradi2g49912	Negative
BdCESA8p-2	Bradi2g49912	Self-active

Table 2. Summary of yeast-two-hybrid interactions between WRIB and putative secondary cell wall transcription factors.

Gene ID	Alias	Family	Interaction
Bradi3g33170	BdWRIB	bHLH	Negative
Bradi5g22920	BdbHLH148	bHLH	Negative
Bradi2g51990	BdbHLH72	bHLH	Negative
Bradi3g37180	BdbHLH93	bHLH	Negative
Bradi3g00300	bZIP45	bZIP	Negative
Bradi3g38840	BdbZIP57	bZIP	Positive
Bradi3g56290	BdbZIP61	bZIP	Positive
Bradi2g54940		BEL1-Like Homeobox	Negative
Bradi1g10050		KNOX Homeobox	Positive
Bradi1g13910	BdHB7	Homeobox	Positive
Bradi1g76970	BdHB9	Homeobox	Self-active
Bradi3g12920		HMG	Negative
Bradi2g48690	BdMADS28	MADS-box	Positive
Bradi1g13680		NFY	Negative
Bradi1g72960		NFY	Negative
Bradi2g46200	BdGNRF	NAC	Negative
Bradi2g05700	BdNAC28	NAC	Positive
Bradi3g13120	BdSWN6	NAC	Positive
Bradi3g13730	BdNAC51	NAC	Self-active
Bradi3g40090	BdNAC60	NAC	Self-active
Bradi1g10470	BdMYB2	MYB	Negative
Bradi2g23710	BdMYB36	MYB	Positive
Bradi3g42430	BdMYB69	MYB	Positive
Bradi2g47590	BdMYB48	MYB	Positive
Bradi2g17980	BdMYB31	MYB	Positive
Bradi2g40620	BdMYB44	MYB	Positive
Bradi2g36730	BdMYB41	MYB	Negative
Bradi1g61400	BdMYB18	MYB	Positive
Bradi5g20130	BdMYB104	MYB	Self-active

APPENDIX A.

NUCLEOTIDE SEQUENCES FOR ALL THE PROMOTER FRAGMENTS USED FOR YEAST-ONE-HYBRID SCREENING

Gene promoter fragment	Promoter fragment sequence
BdCADp-1	ACATAATTCGCGGGATCAATTTCCACCTCCGGGCAATCGAGCGAGATGTGAAT ATCTGATCCCACGACAACTTCCACACACAGGCTGAGATGATATTTTTTTTTCAT CCTCTGCACTAAAAAAGAAAAGCTTAAGCTAGCCACAGAAGATCCAGCCGCAC AATGATAGAAAACGCGCCCCACCTGATCACGGCTGCCGCTGGCAGTCAGTTTT GGCGCACCGTCACGGCTTGATCCCCTCCGGAACTTTCCCTATATATTGCTCTCCC CAGCCCACCACAGTACGAGCACCCAAGAGAGCCGAGAGAGATCGCCTACTCCC AAATCCAGCGCTTTCTACTCTCATCTCTCGGTTCTCTCGCCGTTCTCCGCTCGGT TGACAGAGGAGAGGATAGA
BdCADp-2	AGATCATGTCCTAGTCCTTCTACCAAACAAATACATCACCTGCTCCCATTCGAC GATGATCATCTTGACTTGACGTAGCAATTAGCATATACCAACGAGCGGGGCCG ATGAAAGAGCTTAACACACCTTCGGTTACGTGCTCGCATTTCATATTTCCACTT GTTAACATATCCTCCCTTAGCTTGGCCTCTCTTGTACACAAGAGGAGAGGGCCA AATTAATTCTCGAATATAAATTGCGCATCCAAACTGTTTGAAAATCAAATCTGC TACTAATAAGAAGGACATGAATACAACAACATAATTCGCGGGATCAATTTCCA CCTCCGGGCAATCGAGCGAGATGTGAATATCTGATCCCACGACAACTTCCACA CACAGGCTGAGATGATATTTTTTTTTCATCCTCTGCACTAAAAAAGAAAAGCT
BdCADp-3	TCTTGCTTTAACAAAAGTTCACGCATTAAGACATTTTTGTTCCTATTGCAAGTAC ATCATGCTAAAGGTGTACTTTAACAACAAAGACACTACATGTTACGATGCATCA ATAAGTTTACATAGATGGGCAGCACACGATATTCTTAACAAATCGCACTCTTAT CTACTCTCGGAAGGAAGAAAAATAGTGCTACATTCCCATCCTTACATTTATGTT TGGTCCTTTCAATATCCGCAACTAGTTTGACGATGATTAGATGTCACATCGGAC GATGCACTCCTATTGGCGCCCATGGTTGGTTGCGATGACCTCGTTAGATCATGT CCTAGTCCTTCTACCAAACAAATACATCACCTGCTCCCATTCGACGATGATCAT CTTGACTTGACGTAGCAATTAGCATATACCAACGAGCGGGGCCGATGAAAGAG CTTAACACACCTTCGGTTACGTGCT
BdCESA4p-1	GCCTGGAGAAGTGGCCGAGCAGTGTTTTGCAGAGATTGGTGATTACTTTGCAA AAGGCCTCAGCTAATGTTGTTTGAGGCAATTTTCTGATCTGTTATGTTGGTTTGC ACTCTGATAGCTTATGGTAAAAGATACAGAAATGTTGAGGTTTTGTATGCCACC AAGTTTTCCTCATACTTGATAGCTTATGACAAGACATCGTTGGGTGCATAATAA TCTATGGATATCACAGCATAAAATATTTGCCGTTTTGTTTAAAACATTTCCCATC AGCATTCCCGAGCTGGCACAACGGAAGCTGGCATACGCACGTATAATCTTCCCT TCCTTTCCTCATTAGATCACGTCCCTGCTTGGATGCCTAGGTACAAGTTGATTTT TGTGC
BdCESA4p-2	GGCACAACGGAAGCTGGCATACGCACGTATAATCTTCCCTTCCTTTCCTCATTA GATCACGTCCCTGCTTGGATGCCTAGGTACAAGTTGATTTTTGTGCGACGCCTC ATTAGAAGATGGACATTGTGAGCCGGTGTGCTCGCCCTACATCTTGTCCCTCGG TGAACAAGAATTTTGTTCACCCAGATTTCTATCTCAGGAGATGGCTCTCCTACC ACACGGCCATTTATACACGCACTGGCTTCACGCTCACTCACCATCCCACCAGAA ACGCTGAGGCGTCACCGCAAACCACCCCTACATCGACGTCTCAATTCACCGCTT GCTTCATTCTTGGTCTTCCGGAAAAAACTTCACCCGTCCCTGGCAGTTCCCCAC GAAACACGCGCGCGCGCGCATCTTTCAGCACATTTTGCTTGCCCCGT
BdCESA4p-3	CCCGTCCCTGGCAGTTCCCCACGAAACACGCGCGCGCGCGCATCTTTCAGCA CATTTTGCTTGCCCCGTCCGCGACATTTCCCCCGCACCTAAAGAAGACGCCGCG CTTGCTTTGCAAGCCATCCCACGCTGTGCTTGTCACGCACCTGACTCGCCTTGC GTTGTTGCCTTGGCTTGTGCTCTAATCAAAGCCGGACTCTCCACCCCACGGTTG GTGCCAGACGCCGCCCCGCCACCTTACCGCGCGCGCCAACACCCCACAGTTCTC

	TCTCAAGCTATATCACCCATTAGCCTCCTCGTTGTCCATGTCCCTTCACTGATAC CTCACCCTTCCTCGGACCAACACCATCAGACACCGGCGCGGCGCATTGTCGCGT GCGTGCGTGCTTGCA
BdCESA7p-1	CGACCGGCCAGCTTAATTAATTTTTTCATAATAAAATAAAAAGGAAAGAGTAC TTTGCAGAGGATACATTTGGTGCTTGGTTTCCTCGTGACGGTTCTCGCGCATGT GGGATATCTGTTACACGATCATAGGCGTTGGTAGGTGGCGGGCCAAAAATTTA AGGTGCCTGGTCACCTTCTCCATATCACTCGGGTCAAACT
BdCESA7p-2	GAAAAGGTGACGCGTCTCGTTTGCTTCGTGCGTGCTCAACCCTTACCCGATGGG CTGAGCCTCAAACACGGGTCGCATGGAAACGGACAGAGATCAAAACTTGAAGG CGAGCGACGACCATTTGATTACCGGAAGCGTACGGCTAGAGAGCGATGGT
BdCESA7p-3	TACGTACAACCCACCGATCACCATTAGTTTTCTCAAAAAATCGAAAAAAGGAG ATGGCGGGAGACCCAAGCTTGGGTTGGTCTGGAAGGGGGTTGGTCAGGACTCA GGAGAGACGCGCGCACCTTGCCTGAGCCTGACAACGGCAGCAGAATCCAGCGC AGCGCACCGGCAAAACCAAACCTTTCGCTTCACTTCGCGGCAACGGGGACTCC ACACCCAACCCGCCAAGCTCCCCCTCACCGTCCGGCCACGTCGATCCACAGACC AAATCACAACTCCTTTCTTCGAACTTCGAACAGCCACCCCTCACCTCTCCCCCA CCCGCCAAAACACCTAACCCTCTCATATAGCCCCCCAGCCCCTGCCACAAGCGA ACCAACTAAGCACCCACACTTAGCCCCCGTCTCCTCTCGCCCTGCGCTGCGCTG CACCGGCGCTGCGGCGGAGA
BdCESA8p-1	GGAGCACGACCTACTCCTACGTTGGAAGAGGTATCGAGGGGACTGTTAGCCGG GCATTTTTGAGTAACTTATCAAGTGACCTTTCGGAGCCCTTTTGAGATCGGATG GAACGGGTCTGCTAGAGAAACGAGGTAAGTATACTCTCTGCATTCTAAGTCGA TCCCTCGAGCTCGAGTCTCACTTGAACGTGTATTTCCGAAAAATTGAAACTGCG AAACTCTAACGCTATTCACTTGGGTTTATTCAAATGAGATTAAATTGTTACATG ACAACTGACCATGGAAATGCATGGTCACTCATGAAACTGTAACCACACACTCTT TGCAACCACGTGAACTTCAAGTACCAGGACACAGACTGCACACCATGAAAACT GAAAAAAGTGCCGCAAAATTCATTCTCCACGGAGCACAGGTCTCCTGCTGAAG CTATTCTCTCGGCCCT
BdCESA8p-2	AAGTGCCGCAAAATTCATTCTCCACGGAGCACAGGTCTCCTGCTGAAGCTATTC TCTCGGCCCTCGCCGTGGTGACTCGCGAGTCCGAAAACGGGCTGTCATCTCAAA CAAGAGCGACGCCTCAACGCGAGCGATTTCTTCCATCTTTCTTCAGTTCTTGCTC TCACCGTCCTGAGGGACCCACCCCACCACACCCCCGTTTCACCAACCGGCGCCC CCGACCTCCGACCCTCCCGCCTTGCAGCTCACGCCCCTCCTCGCCGCGGTCACC TTCGCTTCGAAACCCCGCAAAAATCCCGCGAGAAACATCTGCCGCACG
BdCESA8p-3	TTCGAAACCCCGCAAAAATCCCGCGAGAAACATCTGCCGCACGCCGCCCCCCT CTTCCTCCCATCCCATGCCCGCTCAGGCGCCCAACCCTTTGCTTCCATTCTTTCC ATCCTCCACCCGCCGGTACGATGCCGACGCCCCAACCCCACACCCACCGGCCTC CTGCCAGCGCCACACCACCGCGCGGCTATGGCTATATCACCACGCATCCAACCT CCGGCACGCTTAGCCTCTCCCTCCCTCCCTCTCTCACTCCCCCCCTACCCGTTCG CCCGCTGCCGCTCGCGATACCCCGGAGCCGCGTTGGGGGAGCGGGCGAGTAGT GCGCGCACGGAGGGAGAGCC
BdCOMTp-1	ATCCTCATGTCGTGTGCATGGGATGGTAACTCCGACAGGATGTTGCACCACCAA CCCTTCGCGACAACAAGTATATCTTTTTTATCCTAACGTGACGTATATATTTGAT CTGAGTATACGCAAAATAAAAAACTATCAGGAAAACAACCCCACTTATCAACA ACAACTACTACTATGATGTAAACACACACATATTTTTCCCCGGTACCACATTTC TCCCTCACCTTTTCTCCCAAAGTCGAAGAAGAAGGGGAAAAAAACTCAGTTGG TGTGGTGTGGTGGTTGGTGAATGCAGAAAAGCCATATAACCCCTCCCACATCCT CCCTCCCAAATCACACCCTCATCTCCTCTCAGTCGCTCACTCACACCAAGAAGG CAAGAACACACCTACCAAGCAGAAAGAAGAAGCAGCCAGCAACCCCCAGCAG CAATTCGATCC
BdCOMTp-2	ACACTTGCTCACGCTTCAACTCCAACTAAACCCTGCATCTGCATGCGTTCCACC CACCCTCCATCACCATCACGAGACCGATCAAACTGAATTTTCCTACTCTCGTCA TTGCTATCTCCATTTCTCACTGGCGCCACTTGTAGGCTGTGGTGCATGTAGATTT TTGCCAGATTATATTTTTAGTGAGAGTGCTTATATTTAGTTCAACTTTTGAGAGG

	GCAGGAACAACCGCCGGTCCCTCTCACGGTCTCACGAAAAAATAAACATTCCA AACGGGGTAACTGTATACTTTGTTTGGTGTCACGTTTCTTTGACCTCTTTATTTT ATCTAATTTTTGAATACCATCTTTCGAATTCTTGAAAGTTTTTCGAGAAATAATG GTTCAGACGATCCTCATGTCGTGTGCATGGGATGGTAACTCCGACAGGATGTTG CACCACCAACCCTTCGCGACAACAAGTA
BdCOMTp-3	ATCCTCAAGTTGAGGACATGGCATAGCTGATCCAAACGAATCCGTAAAGACCT TAACCTAAAAGTGAAATGATAACATGTTGTCAGCAGGTCAAAATTAAAACCAG GCTCATGTCAAAATCTTGAGAGAAATTTTAGTTTAGGAGTTGAGCCAGGGATCA AATTCAGAGACCAAAAGTATCCTTTTTCTTCTTATTTTCCGTTTTGTCTGATCCT GACGACGGGTGTATAGGCTATGATGACAAGGAATCCGGACTTGAAAAATGAAA ACTTGTCGACCGCTATCACTGACCAAGCGTGACACACATTGCTGGCCACTTGAT CACACTTGCTCACGCTTCAACTCCAACTAAACCCTGCATCTGCATGCGTTCCAC CCACCCTCCATCACCATCACGAGACCGATCAAACTGAATTTTCCTACTCTCGTC ATTGCTATCTCCA

APPENDIX B.

PEPTIDE SEQUENCES FOR ALL THE GENES USED FOR YEAST-TWO-HYBRID SCREENING

Gene	Protein sequence
Bradi1g13980	MGDASRPLGVDDDLMELLWCNGHVVMQSQTHRKLPPRPEKAAAAAAVMQEDE AGLWFPFSHADSLDKDIFSDLFCEAVPQAVGIKPDCYGDGNGSKSSDAPSELMPPP KSTMADGGELSDLVQARSTGKAAAAAMEQEGASASSFCGSSNQVQVQHAGRVQ SAGTAAYGSSARLQSAVGSGINANGRGREATVASSSGRSNGCFTNTTTTSTEPTSA SLRSSSKRKRLDSRTEDYSESPSEDAESESLALIERKPPLKLPTARRSRAAEVHNLS ERRRRDRINEKMKALQELIPHCNKTDKASMLDEAIEYLKTLQMQVQMMWMGSG MAPPAVMFPGMHQYLPRRMPSFMAPPPAAAAAAQSLPDHYAHFLGVNHHLQPP SHHHQHYAAQGMGYYPLGAKAVQQSPALPIHHVHSTANGATPAPAAAAAATNS NTPGNGMHPNRI
Bradi5g21950	MEDGRAARRMSSAPTTRRSRSADFHNFSERRRRDRINEKLKALQELLPNCTKTDK VSMLDEAIDYLKSLQLQLQMLVMGKGMAPVVPPELQQYMHYITADPAQMHMPP LRPTSEPPRPFQITHAGGGGGRPERQSNVESDFLSQMQNLNPSEPPHNFLRPPKLQL YTPEQRGTGLGSSSGHNGGWNIPERNSSYNFME
Bradi1g58230	MEGGGELMELLWQDGAVVAQAQRRCSQSGAGASGVTGEGDAAAWLAPDGGG GRDLYSQLWRSIADADGAGAFVGSSSRTGEAAAGSSFCGSNAVAPPPPLLPSPEA EAEEEEEPGASSAAGGHALLLKRGRDELDDSRCEVVFFFPFLLSGKDLQDADDCE AVDETRTSRRPAGKRRARAAEVHNQSERRRRDRINEKMKALQELVPHCNKSDKA SILDEAIEYLKSLQLQVQIMWMTTGMAPMMFPGAHQLMPQMAMGLNPACMPTA QSLSQLQRVAPFMNNPLPNQMPQVQPPATDFPNVSNQMPNDGVCEPTNPFLHPN DTLAAATQGMFSYGSQRAQQNEIHELMASTAIPASGVCPPSSADTGT
Bradi1g06670	MDTTDLVELLWHNGGVVAHSQRPAAPNPSAAAAADHGGQSTSGLTGEETAAWF PDAALEDDDMYAQLWHSVTTAPDGGGLPAGPSSRPPPGDLAHQTAAAARPPMSS SWTGDLCSTFCGSNQGPVVPAGGGEGSAALPSEGTRGTSTRDGAGTFTGTSSSGG SGSNFGASGLPSESTHGGHKRKGRGRDDSDSRSEDAEFEATEETKSSRRHGSKRRS RAAEVHNQSERRRRDRINEKMRSLQELIPHCNKADKASILDEAIEYLKSLQMQLQI MWMTTGMAPMMFPGAHQFMPPMAMGMNSACMPAAQGLNQMSRVPFINHSLP NHIPMNSSPAMNPMYVANQMQNIHLREASNHFQVAPQVTGPYAYASQVAQQNQ IPEVPDSTVVPTSGAGQPPTSDGI
WRIB	MPEPDQYDEVAELTWEKGNIFWQGLLSRPAPIKYPPPSAAAPMHASPGDRREQTL EAVVGEAAARSSSHSHHLAPNWLAVGVDQVAAGVVPCAAGDADEAEEDARVR NDNKRARRLQACAASQGSAAAPGAARDSPTLLTMEPCGFTATTTNNSTSLDLDH GSPEETTENTSFGGGAASDSRYFSRRDGLCDEAETVVIKEEPPMRMRSSISTKRSR AAAIHNESERKRRDRINQKMQTLQKLVPNSSKTDKASMLDEVIEHLKQLQAQVQ MMSRMGSMMMPMGMAMAPPLQMSVMANMAQMAQMAQMGLGMMNMAAQP AAYPGLASPMNIMQPPNPFVPIQPWDAAGDRQKQPAGIAAVPAYSAFLACQAAA QQQQQAQPNGMEAYNRMVAMYQKLSQQQSQPGNSKQ
Bradi1g48400	MEDFEGGRGAVRELVLMQQERRRRREEEEDELRRQMFGPVVGGAAAFHSASAL AQHHHQQQQQQAAAADCGELGGGFYESEAGGSSEPEPHSSERPRGGSGSKRTRA AEVHNLSEKRRRSRINEKMKALQSLIPNSNKTDKASMLDEAIEYLKQLQLQVQML SMRNGVYLNPSYLSGALEPMQASQMFAALGVGGRNVTAANPGGVVPPVNQNTG AHHSFDPMNSPPQNQQPPLVLPSCPNATIPEPSFHLGTSQSHLRPFQLPESSEMVFR GEIMPKHQITLAQDRANLPGNKMDSVRQEPPMSNTDHFDGCSRSKEHPQDIIPTNT RHA
Bradi3g54640	MMDGGMDQGRFDLIMRHQQQQQHQGFGTMAGLAGAPCDSEEALGSSESEPAGR PARARGKRARAAEVHNLSEKRRRCRINEKMKALQSLVPNSSKTDKASMLDDAIE YLKQLQLQVQMLSMRNGLYLPQVNLPVGAPEPPAAPQMPATVNQNSIEASNPLV ALLPMNQISGAQHSFDPPNHDLRQHHEPFVLSGVPCTTTRGPRFPPGSSRPDLESL QLTVSAEMILQENVMLEHRPSCTQHAVSVPGHDMKLVRQETPVPRPDHPGGCSI WKRQSQEMMPKTTESFLFMPHLHRFQSSDADRGLRSGSK
Bradi2g11100	MSDGNEFAELLWENGQAVVHGRRKHPQPSFQPFGTGSSGVQEKHPGGAGDNMM AFTKAAGVFGGMGIHDLASGLQHENGDDDAVPWIHYPIMEDDNNNNAPALTTA

	DYSSDFFSELQEAAANLGSLPPSNHSTNNNRGTPVAGSSRAASKEIQGLSALTTRT AEPQAELAAAKQPRPSDSVMNFSLFSRPAALARATLQSAHRPQGTDKVPNAAAN NRMESTVIQTTSGPRTGPVFVDQRAAWPQQKEVRFAPAPALTASVGNLQQEMAR DKLSNNRVVHKDAARKAPDATVTTSSVCSGNGIVNDEPWHQQKRKIQAECSASQ DEDLDDESGALLRSTNRSMKRSRTAEVHNLSERRRRDRINEKMRALQELIPNCNK IDKASMLDEAIEYLKTLQLQVQMMSMGTGLCIPPMLLPPAMQHLQLSQMAHFPH LGMGLGYGMGVFDMNNTGTIPVSPMPGAHFPYQMIPGAAAQGLGIPGANAMQM FGIPGQAIPSSGSSIPPFTSLAGLLVRQNLAPEVSGAMPSMVQEQQQGTANQQQQS LNKEAKQ
AtPIF5	MEQVFADWNFEDNFHMSTNKRSIRPEDELVELLWRDGQVVLQSQARREPSVQVQ THKQETLRKPNNIFLDNQETVQKPNYAALDDQETVSWIQYPPDDVIDPFESEFSSH FFSSIDHLGGPEKPRTIEETVKHEAQAMAPPKFRSSVITVGPSHCGSNQSTNIHQAT TLPVSMSDRSKNVEERLDTSSGGSSGCSYGRNNKETVSGTSVTIDRKRKHVMDAD QESVSQSDIGLTSTDDQTMGNKSSQRSGSTRRSRAAEVHNLSERRRRDRINERMK ALQELIPHCSRTDKASILDEAIDYLKSLQMQLQVMWMGSGMAAAAAAAASPMM FPGVQSSPYINQMAMQSQMQLSQFPVMNRSAPQNHPGLVCQNPVQLQLQAQNQI LSEQLARYMGGIPQMPPAGNQMQTVQQQPADMLGFGSPAGPQSQLSAPATTDSL HMGKIG
AtPIF4	MEHQGWSFEENYSLSTNRRSIRPQDELVELLWRDGQVVLQSQTHREQTQTQKQD HHEEALRSSTFLEDQETVSWIQYPPDEDPFEPDDFSSHFFSTMDPLQRPTSETVKPK SSPEPPQVMVKPKACPDPPPQVMPPPKFRLTNSSSGIRETEMEQYSVTTVGPSHCG SNPSQNDLDVSMSHDRSKNIEEKLNPNASSSSGGSSGCSFGKDIKEMASGRCITTD RKRKRINHTDESVSLSDAIGNKSNQRSGSNRRSRAAEVHNLSERRRRDRINERMK ALQELIPHCSKTDKASILDEAIDYLKSLQLQLQVMWMGSGMAAAAAASAPMMFPG VQPQQFIRQIQSPVQLPRFPVMDQSAIQNNPGLVCQNPVQNQIISDRFARYIGGFPH MQAATQMQPMEMLRFSSPAGQQSQQPSSVPTKTTDGSRLDH
At1g09530	MPLFELFRLTKAKLESAQDRNPSPPVDEVVELVWENGQISTQSQSSRSRNIPPPQA NSSRAREIGNGSKTTMVDEIPMSVPSLMTGLSQDDDFVPWLNHHPSLDGYCSDFL RDVSSPVTVNEQESDMAVNQTAFPLFQRRKDGNESAPAASSSQYNGFQSHSLYGS DRARDLPSQQTNPDRFTQTQEPLITSNKPSLVNFSHFLRPATFAKTTNNNLHDTKE KSPQSPPNVFQTRVLGAKDSEDKVLNESVASATPKDNQKACLISEDSCRKDQESE KAVVCSSVGSGNSLDGPSESPSLSLKRKHSNIQDIDCHSEDVEEESGDGRKEAGPS RTGLGSKRSRSAEVHNLSERRRRDRINEKMRALQELIPNCNKVDKASMLDEAIEY LKSLQLQVQIMSMASGYYLPPAVMFPPGMGHYPAAAAAMAMGMGMPYAMGLP DLSRGGSSVNHGPQFQVSGMQQQPVAMGIPRVSGGGIFAGSSTIGNGSTRDLSGS KDQTTTNNNSNLKPIKRKQGSSDQFCGSS
At4g28800	MQFDESDARVWIWFEIRREDDIVELLWQSGQVVGTNQTHRQSYDPPPILRGSGSG RGEENAPLSQPPPHLHQQNLFIQEGEMYSWLHHSYRQNYFCSELLNSTPATHPQSS ISLAPRQTIATRRAENFMNFSWLRGNIFTGGRVDEAGPSFSVVRESMQVGSNTTPP SSSATESCVIPATEGTASRVSGTLAAHDLGRKGKAVAVEAAGTPSSGVCKAETEP VQIQPATESKLKAREETHGTEEARGSTSRKRSRTAEMHNLAERRRREKINEKMKT LQQLIPRCNKSTKVSTLDDAIEYVKSLQSQIQGMMSPMMNAGNTQQFMPHMAM DMNRPPPFIPFPGTSFPMPAQMAGVGPSYPAPRYPFPNIQTFDPSRVRLPSPQPNPV SNQPQFPAYMNPYSQFAGPHQLQQPPPPPFQGQTTSQLSSGQASSSKEPEDQENQP TA
At4g28815	MMIISSQILLLFGFKLFFETRGEDDIVELLCKIGQTQIPSSDPLPILRGSGSGGREENT PLPPPLPHQNLFIQEDEMSSWPHHPLRQDYLCSELYASTPAPHPQSSVSLAPPPPKP PSSAPYGQIIAPRSAPRIQGTEEARGSTSRKRSRAAEMHNLAERRRREKINERMKT LQQLIPRCNKSTKVSMLEDVIEYVKSLEMQINQFMPHMAMGMNQPPAYIPFPSQA HMAGVGPSYPPPRYPFPNIQTFDPSRVWLQSPQPNPVSNQPQMNPYGQFVGHHQ MQQSLPPPLQVILSQYPLCLFLCSNK
At4g28811	MGEDDIVELLWNGQVVRTSQPQRPSSGKPSPTPPILRGSGSGSGEENAPLPLPLLQP PRPLHHQNLFIREEEMSSWLHYSYTGVTSTPATHPQSSVSLPPPPPIAPSEDDVVEL LWKSGQVVQSIQTQRPIPPPIFRGSGSGGGEETVLPLPPLHPSHQNIFIQEDEMASW LYHPLRQDYFSSGVASTSATRPQSSASLAPTPPPPSVPYGQIPVERRTENFMNFLRL RGNIFSGGRVEAGPVVIESTQIGSSATPSSSAAESCVIPATHGTESRAAAITGVSRTF

	AVPGLGRRGKEVATETAGTSYSGVNKAETERVQIQPERETKITEDKKREETIAEIQ GTEEAHGSTSRKRSRAADMHNLSERRRRERINERMKTLQELLPRCRKTDKVSMLE DVIEYVKSLQLQIQMMSMGHGMMPPMMHEGNTQQFMPHMAMGMKGMNRPPP FVPFPGKTFPRPGHMAGVGPSYPALRYPFPDTQASDLSRVHVPSLHSNPVPNQPRF PAYINPYSQFVGLHQMQQPPLPLQGQPTSQPSFSHASTSK
At4g28790	MTWKPKMLILSHDLISPEKYIMGEDDIVELLGKSSQVVTSSQTQTPSCDPPLILRGS GSGDGEGNGPLPQPPPPLYHQQSLFIQEDEMASWLHQPNRQDYLYSQLLYSGVAS THPQSLASLEPPPPPRAQYILAADRPTGHILAERRAENFMNISRQRGNIFLGGVEAV PSNSTLLSSATESIPATHGTESRATVTGGVSRTFAVPGLGPRGKAVAIETAGTQSW GLCKAETEPVQRQPATETDITDERKRKTREETNVENQGTEEARDSTSSKRSRAAIM HKLSERRRRQKINEMMKALQELLPRCTKTDRSSMLDDVIEYVKSLQSQIQMFSMG HVMIPPMMYAGNIQQQYMPHMAMGMNRPPAFIPFPRQAHMAEGVGPVDLFREN EETEQETMSLLLREDKRTKQKMFS
AtPIL5	MHHFVPDFDTDDDYVNNHNSSLNHLPRKSITTMGEDDDLMELLWQNGQVVVQN QRLHTKKPSSSPPKLLPSMDPQQQPSSDQNLFIQEDEMTSWLHYPLRDDDFCSDLL FSAAPTATATATVSQVTAARPPVSSTNESRPPVRNFMNFSRLRGDFNNGRGGESG PLLSKAVVRESTQVSPSATPSAAASESGLTRRTDGTDSSAVAGGGAYNRKGKAVA MTAPAIEITGTSSSVVSKSEIEPEKTNVDDRKRKEREATTTDETESRSEETKQARVS TTSTKRSRAAEVHNLSERKRRDRINERMKALQELIPRCNKSDKASMLDEAIEYMK SLQLQIQMMSMGCGMMPMMYPGMQQYMPHMAMGMGMNQPIPPPSFMPFPNM LAAQRPLPTQTHMAGSGPQYPVHASDPSRVFVPNQQYDPTSGQPQYPAGYTDPY QQFRGLHPTQPPQFQNQATSYPSSSRVSSSKESEDHGNHTTG
AtPIL1	MEAKPLASSSSEPNMISPSSNIKPKLKDEDYMELVCENGQILAKIRRPKNNGSFQK QRRQSLLDLYETEYSEGFKKNIKILGDTQVVPVSQSKPQQDKETNEQMNNNKKKL KSSKIEFERNVSKSNKCVESSTLIDVSAKGPKNVEVTTAPPDEQSAAVGRSTELYF ASSSKFSRGTSRDLSCCSLKRKYGDIEEEESTYLSNNSDDESDDAKTQVHARTRKP VTKRKRSTEVHKLYERKRRDEFNKKMRALQDLLPNCYKDDKASLLDEAIKYMRT LQLQVQMMSMGNGLIRPPTMLPMGHYSPMGLGMHMGAAATPTSIPQFLPMNVQ ATGFPGMNNAPPQMLSFLNHPSGLIPNTPIFSPLENCSQPFVVPSCVSQTQATSFTQ FPKSASASNLEDAMQYRGSNGFSYYRSPN
AtPIL2	MMFLPTDYCCRLSDQEYMELVFENGQILAKGQRSNVSLHNQRTKSIMDLYEAEY NEDFMKSIIHGGGGAITNLGDTQVVPQSHVAAAHETNMLESNKHVDDSETLKASS SKRMMVDYHNRKKIKFIPPDEQSVVADRSFKLGFDTSSVGFTEDSEGSMYLSSSL DDESDDARPQVPARTRKALVKRKRNAEAYNSPERNQRNDINKKMRTLQNLLPNS HKDDNESMLDEAINYMTNLQLQVQMMTMGNRFVTPSMMMPLGPNYSQMGLA MGVGMQMGEQQFLPAHVLGAGLPGINDSADMLRFLNHPGLMPMQNSAPFIPTEN CSPQSVPPSCAAFPNQIPNPNSLSNLDGATLHKKSRKTNR
AtHFR1	MSNNQAFMELGWRNDVGSLAVKDQGMMSERARSDEDRLINGLKWGYGYFDHD QTDNYLQIVPEIHKEVENAKEDLLVVVPDEHSETDDHHHIKDFSERSDHRFYLRN KHENPKKRRIQVLSSDDESEEFTREVPSVTRKGSKRRRRDEKMSNKMRKLQQLVP NCHKTDKVSVLDKTIEYMKNLQLQLQMMSTVGVNPYFLPATLGFGMHNHMLTA MASAHGLNPANHMMPSPLIPALNWPLPPFTNISFPHSSSQSLFLTTSSPASSPQSLH GLVPYFPSFLDFSSHAMRRL
AtPIF7	MSNYGVKELTWENGQLTVHGLGDEVEPTTSNNPIWTQSLNGCETLESVVHQAAL QQPSKFQLQSPNGPNHNYESKDGSCSRKRGYPQEMDRWFAVQEESHRVGHSVTA SASGTNMSWASFESGRSLKTARTGDRDYFRSGSETQDTEGDEQETRGEAGRSNG RRGRAAAIHNESERRRRDRINQRMRTLQKLLPTASKADKVSILDDVIEHLKQLQA QVQFMSLRANLPQQMMIPQLPPPQSVLSIQHQQQQQQQQQQQQQQQQQFQMSLL ATMARMGMGGGGNGYGGLVPPPPPPPMMVPPMGNRDCTNGSSATLSDPYSAFF AQTMNMDLYNKMAAAIYRQQSDQTTKVNIGMPSSSSNHEKRD
At5G62610	MDPPLVNDSSFSAANPSSYTLSEIWPFPVNDAVRSGLRLAVNSGRVFTRSEHSGNK DVSAAEESTVTDLTAGWGSRKTRDLNSEDDSSKMVSSSSSGNELKESGDKKRKL CGSESGNGDGSMRPEGETSSGGGGSKATEQKNKPEPPKDYIHVRARRGQATDRH SLAERARREKISEKMTALQDIIPGCNKIIGKALVLDEIINYIQSLQRQVEFLSMKLEV VNSGASTGPTIGVFPSGDLGTLPIDVHRTIYEQQEANETRVSQPEWLHMQVDGNF NRTT

At4g00050	MSQCVPNCHIDDTPAAATTTVRSTTAADIPILDYEVAELTWENGQLGLHGLGPPR VTASSTKYSTGAGGTLESIVDQATRLPNPKPTDELVPWFHHRSSRAAMAMDALVP CSNLVHEQQSKPGGVGSTRVGSCSDGRTMGGGKRARVAPEWSGGGSQRLTMDT YDVGFTSTSMGSHDNTIDDHDSVCHSRPQMEDEEEKKAGGKSSVSTKRSRAAAIH NQSERKRRDKINQRMKTLQKLVPNSSKTDKASMLDEVIEYLKQLQAQVSMMSR MNMPSMMLPMAMQQQQQLQMSLMSNPMGLGMGMGMPGLGLLDLNSMNRAA ASAPNIHANMMPNPFLPMNCPSWDASSNDSRFQSPLIPDPMSAFLACSTQPTTME AYSRMATLYQQMQQQLPPPSNPK
AtALC	MGDSDVGDRLPPPSSSDELSSFLRQILSRTPTAQPSSPPKSTNVSSAETFFPSVSGGA VSSVGYGVSETGQDKYAFEHKRSGAKQRNSLKRNIDAQFHNLSEKKRRSKINEK MKALQKLIPNSNKTDKASMLDEAIEYLKQLQLQVQTLAVMNGLGLNPMRLPQVP PPTHTRINETLEQDLNLETLLAAPHSLEPAKTSQGMCFSTATLL
AtSPT	MISQREEREEKKQRVMGDKKLISSSSSSSVYDTRINHHLHHPPSSSDEISQFLRHIFD RSSPLPSYYSPATTTTTASLIGVHGSGDPHADNSRSLVSHHPPSDSVLMSKRVGDF SEVLIGGGSGSAAACFGFSGGGNNNNVQGNSSGTRVSSSSVGASGNETDEYDCES EEGGEAVVDEAPSSKSGPSSRSSSKRCRAAEVHNLSEKRRRSRINEKMKALQSLIP NSNKTDKASMLDEAIEYLKQLQLQVQMLTMRNGINLHPLCLPGTTLHPLQLSQIR PPEATNDPLLNHTNQFASTSNAPEMINTVASSYALEPSIRSHFGPFPLLTSPVEMSR EGGLTHPRLNIGHSNANITGEQALFDGQPDLKDRIT

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