Characterization of proteins involved in MYB transcription factor
complexes that regulate suberin deposition in *Arabidopsis thaliana*

by

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ABSTRACT

Terrestrial plants deposit cell wall-associated, glycerolipid-based polymeric barriers in specific tissue layers as protection against various environmental stresses (e.g. drought, salinity, toxins, and pathogens). One of these barriers, suberin, is found in the cell walls of various underground and aerial tissues, including root endodermis, tuber periderms, and bark (cork). Suberin influences water uptake and retention, ion transport, and gas exchange within roots. A set of myeloblastosis (MYB)-type transcription factors (TFs) has been found to regulate suberin production in the model plant *Arabidopsis thaliana*. The functions of MYB TFs are known to be influenced by protein-protein interactions with other TFs at the promoters of target genes. Hence, the main focus of this study was to characterize protein-protein interactions involving MYB TFs that regulate suberin deposition in root endodermis. I used a targeted yeast two-hybrid assay to identify a physical interaction between *Arabidopsis* MYB92 and MYB93 with bHLH111 (a basic helix-loop-helix TF). In addition, the root expression pattern of the *bHLH111* gene, as visualized by a promoter : reporter gene (GUS) assay, was consistent with a role in regulating suberin deposition in the endodermis. Knock-out mutations of *bHLH111* and the related *bHLH112* gene were found to have reduced levels of suberin in young *Arabidopsis* roots. Altogether, these findings provide evidence for bHLH proteins working together with MYB TFs to regulate suberin production in the root endodermis of *Arabidopsis*.

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LIST OF ABBREVIATIONS

aa: amino acids

ABC: ATP-binding cassette

ABRC: Arabidopsis Biological Resource Center

ASFT: aliphatic suberin feruloyl transferase

BD: binding domain

bHLH: basic helix-loop-helix

BiFC: bi-molecular fluorescence complementation

bp: base pairs

C+ : positive control

C- : negative control

cDNA: complimentary deoxyribonucleic acid

ChIP: chromatin immunoprecipitation

co-IP: co-immunoprecipitation

CYP: cytochrome P450 monooxygenase

DCA: dicarboxylic fatty acid

DDO: double drop-out yeast media

DNA: deoxyribonucleic acid

DW: dry weight

ER: endoplasmic reticulum

FAE: fatty acid elongation

FAR: fatty acyl reductase

FAS: fatty acid synthase

FID: flame ionization detector

GFP: green fluorescent protein

GUS: β -glucoronidase

HCA: hydroxycinnamate

HTH: helix-turn-helix

KO: gene knock-out

LC: long-chain

MBW: MYB/bHLH/WDR

ME: methyl ester

MS: Murashige and Skoog

MYB: myeloblastosis related protein

OFP: ovate family protein

OPL: omega-pentadecanolactone

PCR: polymerase chain reaction

PDO: pentuple drop-out yeast media

PM: plasma membrane

PPI: protein-protein interaction

PTM: post-translational modification

QDO: quadruple drop-out yeast media

RT-PCR: reverse transcriptase polymerase chain reaction

RNA: ribonucleic acid

SD: synthetic drop-out yeast media

SDO: single drop-out yeast media

SDS: sodium dodecyl sulphate

seq: sequencing

TAD: transcriptional activation domain

TAIR: The *Arabidopsis* Information Resource

TDO: triple drop-out yeast media

TEM: transmission electron microscopy

TF: transcription factor

VLC: very long-chain

WDR: Cytosolic repeat protein

WT: wild-type

Y1H: yeast one-hybrid

Y2H: yeast two-hybrid

Y3H: yeast three-hybrid

TZ: tetrazolium red

CHAPTER 1: INTRODUCTION

Terrestrial plants produce protective lipid-based barriers that modify the cell walls of tissue layers located at environmental interfaces. These structures are organized into chemically complex glycerolipid polymers and associated waxes, with cuticle (cutin + waxes) coating aerial surfaces and suberin + waxes deposited in the walls of various internal and external tissues (Kolattukudy, 1984)(Bernards, 2002)(Vishwanath *et al.*, 2015). Both cuticle and suberin are involved in water relations and gas exchange. These layers also provide physical protection from biotic (e.g. microbial pathogens) and abiotic (e.g. high salinity, drought) stresses (Enstone *et al.*, 2003)(Ranathunge *et al.*, 2011)(Graça, 2015). Additionally, the enzymes involved in suberin biosynthesis are of industrial value, since the chemical compositions of their products consists largely of fatty acid derivatives that can be used for production of plasticizers in resins, bioplastics, natural insecticides, biopharmaceutical precursors, and novel biofuels (Rowland and Domergue, 2012) (Kosma *et al.*, 2014). Potentially, genetically engineered organisms (e.g. bacteria or plants) specialized in suberin monomer synthesis could enzymatically produce large amounts of these unique lipid molecules to be used for industrial or commercial purposes.

Within the past decade, suberization has been studied extensively in the model eudicot plant, *Arabidopsis thaliana*, largely due to its rapid growth rate, small size, and adaptability to different types of experiments. Findings from suberin research in *Arabidopsis* may then be tested in crop species, in order to observe the effects of enhanced suberization on growth, yield and stress tolerance abilities. Translating

our knowledge of the suberin deposition process from a model organism to major crops (e.g. rice, maize, wheat, rapeseed) could allow for the future generation of genetically modified crops that are more stress resistant, via enhancement of suberin deposition at certain tissue interfaces, in an effort to develop more sustainable global agriculture. Environmental stress can cause major yield loss of harvested crops, and improvement of stress tolerance can mitigate, and potentially eliminate, the problem.

1.1. Suberin: structure and functions

Suberin is a glycerol-based biopolyester that is deposited in the apoplastic space and adjacent to the outside face of the plasma membrane. Suberin is found as such in the walls of cells in various organs, such as roots, bark, seed coats, and wounded tissues. Aliphatic suberin is composed of a complex mixture of long-chain (LC; C16-C20) and very-long-chain (VLC; C22-C26) fatty acids and their derivatives linked with glycerol and phenolics (mostly ferulate) (Graça, 2015). The aliphatic component of suberin includes LC and VLC α,ω -dicarboxylic fatty acids (DCAs), ω -hydroxy fatty acids, and primary fatty alcohols (Graça, 2015). Aliphatic suberin monomers are typically saturated or mono-unsaturated. The insoluble polyphenolic content of suberin consists of monolignols and polymerized phenolics such as ferulate, tyramine, coumarate, and other hydroxycinnamate (HCA) monomers. Polyphenolics are especially abundant in wounded tissues, where the ratio of polyphenolics to aliphatic suberin is 2:1, as observed in potato tuber wound periderm (Mattinen *et al.*, 2009).

Suberin protects plants against biotic stresses (e.g. pathogen invasion) and abiotic stresses (e.g. root drying) (Enstone *et al.*, 2003). Suberin is involved with the abiotic stress response in roots through its role in the movement of water, ions, and gases (Ranathunge *et al.*, 2011). Suberin has a major role ion homeostasis and ion translocation within root tissues, as shown in crop species such as barley (*Hordeum vulgare*)), where manganese levels in the soil can affect the extent of suberization, and subsequently the transport of other ions (calcium, potassium, sodium, sulfur, etc.) into the stele from the cortex (Barberon *et al.*, 2016) (Chen *et al.*, 2019).

Suberin is a chemically complex polyester found in three main structural forms. There are several hypothesized models of “sealant” suberin in terms of Casparian band structure and development. Contradictions in the literature can make it difficult to form a clear consensus of how suberin relates to Casparian bands (Naseer *et al.*, 2012) (Geldner, 2013) (Graça, 2015) (Andersen *et al.*, 2015) (Barberon, 2017). In one working model, suberin seals the spaces in between root endodermal or root exodermal cells, with the incrustation of suberin, lignin, and other components forming the Casparian bands that are localized in the radial and transverse directions in most vascular plants, and form cylinders within the root endodermis (and root exodermis when present) (Enstone *et al.*, 2003). Suberin (polyaliphatic and polyphenolic domains) in the form of Casparian bands is thought to act as a hydrophobic barrier impeding apoplastic transport of water and preventing backflow of ions, and they are also part of tertiary dermis walls, hypothesized to be involved in mechanical support of the root (Enstone *et al.*, 2003). An alternate model is that sealant suberin is entirely separate from the Casparian

bands, wherein the bands are composed of a lignin-like polymer and suberin surrounds root endodermal cells as a late differentiated state of root endodermis (Andersen *et al.*, 2015). It is important to note that the latter model is limited, since all results supporting this model have been obtained only from *Arabidopsis*. Thus, the model needs to be further supported by studies with other plant species.

Diffuse suberin is deposited within the intermicrofibrillar channels of young root epidermis (Peterson and Cholewa, 1998). Suberin deposited as sheet-like structures along the primary cell walls, with the alternating sheets in walls of the root endodermis (and root exodermis for monocots), is termed “suberin lamellae”. The sheets of suberin lamellae alternate between light and dark bands when viewed with transmission electron microscopy (TEM) (Figure 1.1). It has been proposed that aliphatic suberin corresponds to the light bands and polyphenolics correspond to the dark bands (Graça, 2015). This alternating pattern could relate to varying degrees of osmium densities fixed onto the thin section of root tissue being analyzed by TEM. Osmium tetroxide (OsO_4) is used as a fixative chemical for TEM samples; OsO_4 reacts with alkene bonds of unsaturated lipids and aromatic compounds, rendering them resistant to extraction by many solvents (Porter and Kallman, 1953). OsO_4 becomes reduced by double-bond sites to a lower oxide or hydroxide that results in a blackened compound that coats the surface of the sample wherever it is bound, but OsO_4 does not interact with saturated lipids (Porter and Kallman, 1953). The suberin lamellae ultrastructure may appear banded due to the fixation process creating denser polyaromatic layers (with more alkene bonds) and less osmium-dense polyaliphatic layers (Figure 1.1). Sheet-like suberin in the root

endodermis develops after the formation of Casparian bands, so the lamellae are not essential for establishing a functional apoplastic barrier (Andersen *et al.*, 2015)(Barberon, 2017). However, suberin lamellae potentially modulate either the apoplastic or transcellular pathways of water/solute uptake from the rhizosphere to the root vasculature, with the suberized endodermal cell layer as a checkpoint for water and nutrients (i.e. polar or charged molecules) (Andersen *et al.*, 2015)(Barberon, 2017).

In one model of sheet-type suberin, the bio-polymer is defined as a polyacylglycerol macromolecule, composed of glycerol subunits successively crosslinked as a polyester to LC and VLC FAs (mainly α,ω -DCAs) with varying degrees of mid-chain modifications, which forms the core structural backbone of suberin lamellae (Figure 1.2). In differing models of suberin structure, the polyacylglycerol may expand into a reticulated and disordered network of random crosslinks (Pollard *et al.*, 2008), or it could be a more ordered structure arising from linearly packed fatty acid chains (Graça and Santos, 2006).

Although the polyphenolic domain of suberin is highly similar to lignified tissues, the difference arises from significant amounts of non-lignin precursors (hydroxycinnamic acids (HCAs) and derivatives) found in suberin that are cross-linked in ways analogous to monolignols (Bernards, 2002). For the polyaliphatic domain, a three-dimensional network of modified fatty acids/alcohols esterified to glycerol-bridging subunits is proposed based on current knowledge of suberin monomer composition. The 'Bernards' model of suberized tissues consists of an HCA-based polyphenolic domain restricted to the primary cell wall, which is

covalently esterified to a glycerol-based polyaliphatic (i.e. polyacylglycerol) domain residing in the space between the cell wall and plasma membrane (Figure 1.3).

Figure 1.1: TEM image of *Arabidopsis thaliana* (4-week-old) root periderm cell showing the presence of suberin lamellae as alternating light and dark bands of sheet-type suberin. PW, primary cell wall; S, suberin; PM, plasma membrane; Cy, cytoplasm. Original figure was modified; TEM was performed by Nayana de Silva (de Silva, 2018).

Figure 1.2: Hypothetical model of sheet-type suberin in the context of suberized cell walls. The core suberin structure is defined as a regularly packed polyacylglycerol polyester macromolecule, forming light translucent lamellae. Aliphatic glycerol subunits are covalently linked to polyphenolics, possibly forming the dark lamellae in alternating bands, which can be seen in Figure 1. Original figure was modified with permission from author (Graça, 2015).

Figure 1.3: Model of suberin structure in potato tuber periderm. The polyphenolic domain is embedded in the primary cell wall, whereas the polyaliphatic domain resides between the cell wall and plasma membrane. The polyaliphatic domain is predominantly composed of modified fatty acids/alcohols esterified to glycerol subunits, which are covalently linked to polyphenolics within the polyaliphatic layers, forming the dark lamellae in alternating bands. Original figure was modified with permission from author (Bernards, 2002).

1.2. Suberin: biosynthesis and deposition

The suberin biosynthetic pathway within plant cells is outlined schematically in Figure 1.4. Suberin precursor biosynthesis involves the collective action of several enzymes in the plastid and the endoplasmic reticulum (ER) of the plant cell, such as fatty acid synthase (FAS), fatty acyl reductases (FARs), cytochrome P450 monooxygenases (CYPs), glycerol-3-phosphate acyltransferases (GPATs), and fatty acid elongase (FAE) complexes, as shown in Figure 1.5 (Vishwanath *et al.*, 2015). Firstly, C16-18 acyl chains are synthesized in plastids and transported to the outer leaflet of the SER membrane to become activated CoA thioesters (fatty acyl-CoAs), then a proportion of these get elongated to C20-C24 CoA by FAE. The LC and VLC products can be oxidized by CYPs to produce α,ω -DCAs and ω -hydroxy fatty acids, which then get linked to glycerol by GPATs to create *sn*2-monoacylglycerols as the core aliphatic monomers of suberin. Additionally, a reductive pathway exists to produce primary fatty alcohols by FARs and these fatty alcohols can be part of the suberin polymer, but most will get linked to hydroxycinnamates (HCAs) to produce soluble alkyl hydroxycinnamates (AHCs), which may be associated with the suberin polymer (Vishwanath *et al.*, 2015).

Synthesized suberin monomers or short oligomers are transported from the ER to the plasma membrane (PM), and then across the PM via specific ATP-binding cassette (ABC) transporters. A certain clade of ABCG half-transporters, are co-expressed with several suberin biosynthesis genes, and have been found to be involved in suberin deposition, specifically ABCG2, ABCG6 and ABCG20 as major candidates (Yadav *et al.*, 2014)(Andersen *et al.*, 2015). Another aspect of the

transport system likely involves secretory vesicles, which have been shown to deliver cuticular lipids from the ER to PM-localized ABC transporters in plant epidermal cells, so this transport system may be similar for suberin-associated lipids (McFarlane *et al.*, 2014). Following biosynthesis of precursors and their subsequent transport across the PM, suberin polymers are formed along the inner cell wall region. Specialized polyester synthases may be involved in the process, as is the case for cutin (Yeats *et al.*, 2013), but these have yet to be identified for suberin. In the cytoplasm, evidence suggests that aliphatic suberin feruloyl transferase (ASFT) esterifies ferulate (in the form of feruloyl-CoA) to ω -hydroxy fatty acids, and these alkyl-ferulates are also deposited into suberin polymers (Molina *et al.*, 2009) (Figure 1.5).

Deposition of suberin lamellae of root endodermal cells begins with an intermittent “patchy” pattern in state I of differentiation (within the differentiation zone), just above the younger undifferentiated region closest to the growing root tip. The gene expression patterns of genes encoding suberin biosynthesis enzymes (e.g. FAR1/FAR4/FAR5, GPAT5, CYP86B1/RALPH) follow the sporadic distribution of patchy suberin in state I root endodermis (Domergue *et al.*, 2010)(Beisson *et al.*, 2007)(Compagnon *et al.*, 2009), hence they are used as indicators for defining the onset of suberization in roots (Naseer *et al.*, 2012)(Andersen *et al.*, 2015). The patchy suberization becomes continuous in differentiated state II root endodermis, moving up toward the mature root. Suberin deposition is usually limited to specialized cell types, but expression of biosynthesis genes can be induced by environmental stresses. The expression patterns of these gene sets are indicative of

transcriptional regulation for the suberin pathway since they reflect the known tissue-specific localization of suberin deposition (Vishwanath *et al.*, 2015).

Currently, there is little information regarding transcriptional regulation of genes involved in the suberin biosynthetic pathway.

Figure 1.4: Schematic overview of the suberin pathway within plant cells. A signal cascade recruits transcription factors (e.g. MYB) to the promoter regions to regulate transcription of genes encoding for suberin biosynthetic enzymes. Transcribed mRNA is exported from the nucleus to the rough endoplasmic reticulum (RER) for translation into enzyme, which are then translocated to the smooth ER (SER). Fatty acid (FA) precursors synthesized in plastids are used to create more complex suberin monomers to shuttle across the plasma membrane (PM) to form the suberin

lamellae between the PM and primary cell wall. AHCs are soluble waxes that are not covalently linked to the polymer, but are likely associated in some way.

Figure 1.5: Schematic overview of the suberin biosynthetic pathway and transport/deposition of suberin on the inner cell wall of a plant cell. Aliphatic suberin precursors, shuttled across the PM via ABCG transporters, are polymerized in the form of sheet-type suberin to form lamellae of the secondary cell wall. Regulatory MYB TFs reside in the nucleus and control transcription of suberin-related genes. Original figure was modified (Vishwanath *et al.*, 2015).

1.3. MYB transcription factors: structure and functions

The myeloblastosis related proteins (MYB) family of TFs are functionally diverse and present in all eukaryotes. In plants, MYB domains are identified by a conserved MYB DNA-binding domain (BD) with typically one to four imperfect repeats. Classification of MYB subfamilies and binding affinities are dependent on the number of adjacent DNA BD repeats (Li *et al.*, 2015). These MYB repeat sequences are each about 52 amino acids in length, and each containing a hydrophobic core of three regularly spaced aliphatic residues (e.g. tryptophan). MYB repeats exist in a helix-turn-helix confirmation, and the N-terminal α -helix in each repeat specifically recognizes target DNA sequences (~6-12 bp) and binds to the major DNA groove (Feller *et al.*, 2011). The R2R3-MYB subfamily is most abundant in plants. R2R3-MYBs have a conserved MYB N-terminal DNA BD and variable C-terminal transcriptional activation domains (TADs) that are often regulated via post-translational modifications (PTMs) (Feller *et al.*, 2011) (Pireyre and Burow, 2015). Although the TAD is named after its activation activity, it is entirely possible that this domain exhibits repression activity as well. Figure 1.6 provides a schematic representation of R2R3-MYB structure, and Figure 1.7 shows a protein sequence alignment.

MYB proteins are among one of the largest groups in plants (~9% of total TFs in *Arabidopsis*), which suggests that there are several specialized functions for MYBs (Riechmann *et al.*, 2000). It has been demonstrated that MYBs regulate transcriptional activity of many biological processes, including seed and floral development, senescence, cell differentiation, stress responses, and secondary

metabolism in plants. Extensive characterization of MYB functions have been gathered from numerous studies with the model plant *Arabidopsis thaliana* (Dubos *et al.*, 2010), in addition to other plant species, such as poplar (Ma *et al.*, 2018) and petunia (Schwinn *et al.*, 2014).

Figure 1.6: Schematic diagram of R2R3-MYB proteins. In plants, MYB domains are identified by a conserved MYB DNA-binding domain (DBD), with R2R3 repeat family being the most abundant (basic protein structure shown in figure). Each MYB repeat is ~52 amino acids (aa) long, with a tryptophan (W) repeat pattern that forms the hydrophobic core of each α -helix. The C-terminal domain contains a transcriptional activation domain (TAD). HTH, helix-turn-helix.

Plants rely on root systems for survival, and as plants grow their root systems increase in structural complexity; lateral roots contribute to this complexity. The endodermis, overlaying the pericycle cell layer, functions in regulation of lateral root emergence, influenced by internal signals and environmental factors. It has been found that the MYB93 TF in *Arabidopsis* (AtMYB93) is an auxin-induced negative regulator of lateral root development. Supporting evidence includes the localized expression of *AtMYB93* transcripts in epidermal regions of lateral root organogenesis, and only during early stages of formation (Gibbs and Coates, 2014) (Gibbs *et al.*, 2014). These studies exemplify the regulation of tissue-specific developmental processes via AtMYB TFs.

In another study, an *AtMYB* homolog (*AtMYB2*) was constructed from a cDNA library isolated from dehydrated *Arabidopsis* rosettes (Urao *et al.*, 1993). Experiments revealed that AtMYB2 levels in vegetative tissues were increased upon dehydration stress, and restored to normal upon rehydration. In addition, *AtMYB2* mRNA expression was induced by salt stress, ABA signals, and seed maturation events. β -glucuronidase (GUS) fusion promoter : reporter gene assays provide further evidence for transcriptional regulation of the *AtMYB2* promoter in relation to abiotic stresses (Urao *et al.*, 1993). This study provides an example of AtMYB TFs involved in stress-related functions, indicated by increased expression levels upon exposure to stressors.

As a non-*Arabidopsis* example, a sugar transporter protein from apple (*Malus domestica*, MdSTP13a) is responsible for the uptake of both sucrose and hexose into

growing pollen tubes of apple flowers. This sugar transporter is under the transcriptional control of MdMYB39L, which binds directly to the promoter site of the *MdSTP13a* gene to activate its expression (Li *et al.*, 2019). Additionally, CsMYBF1 from sweet orange citrus tree (*Citrus sinensis*) has been identified in the positive regulation of flavonol and HCA biosynthesis genes, demonstrated by RNAi suppression studies and transactivation assays in citrus and tomato varieties (Liu *et al.*, 2016). This could have implications for suberin studies, since certain phenylpropanoid pathway genes in *Arabidopsis* are hypothesized to be under the control of MYB TFs, supported by the evidence that MYBs are key regulators of suberin-associated genes, in addition to various other developmental and stress-induced genes (Kosma *et al.*, 2014) (Li *et al.*, 2015) (Legay *et al.*, 2016) (Hu, 2018) (Klein, 2019).

1.4. Suberin-related MYBs

Recently, MYBs have been implicated in the regulation of the suberin biosynthetic pathway. Figure 1.8 illustrates the phylogenetic relationship between suberin-related AtMYB proteins, based on the Maximum Likelihood method.

Figure 1.8: Phylogenetic relationship of select AtMYB TFs. Protein sequences used in the analysis are conserved R2R3 repeats (92 amino acids long). Tree was constructed using the Maximum Likelihood method based on the JTT matrix-based model with 1000 bootstrap replicates and discrete Gamma distribution (10

categories) to model evolutionary rate differences among sites (MEGA7 software). Numbers beside branches represent the percentage of trees in which the associated proteins clustered together.

In studies of *A. thaliana* seed coats, knockout mutants of MYB107 (*myb107*) were shown to have increased seed coat permeability and this was correlated with decreased levels of suberin monomers (Gou *et al.*, 2017). The *myb107* mutants also have alteration of suberin biosynthetic gene expression, affecting the overall composition of suberin in their seed coats. In addition, transcripts of *AtMYB107* are co-expressed with seed suberin biosynthetic genes, and *AtMYB107* is nuclear localized. The interaction of *AtMYB107* with its biosynthetic gene promoters was observed using yeast one-hybrid (Y1H) and chromatin immunoprecipitation (ChIP) coupled with quantitative PCR (qPCR). For Y1H, promoter : reporter gene constructs were designed with selected biosynthetic suberin genes (*FAR1*, *FAR4*, *FAR5*, *GPAT5*, *HHT*, *FACT*) and placed upstream of a *LacZ* reporter gene. Yeast (*Saccharomyces cerevisiae*) containing suberin gene constructs grew to full coloration on selective media when *AtMYB107* was expressed. To support these findings, ChIP/qPCR was also performed. MYB107:GFP fusion constructs were designed for each tested suberin gene (*FAR1*, *FAR4*, *FAR5*, *GPAT5*, *HHT*, *FACT*), and promoter-specific complementary primers were used to amplify promoter regions for qPCR analysis. It was found that the promoter regions of the tested genes (amplified by PCR) were enriched in MYB107:GFP immunoprecipitates, indicative of specific binding of *AtMYB107* to the promoters of suberin biosynthetic genes. The collective results

indicate that AtMYB107 is directly involved in transcriptional regulation of suberin biosynthetic genes in seed coats (Gou *et al.*, 2017). A study with similar results includes both AtMYB107 and AtMYB9, which were both shown to regulate suberin assembly in seed coats (Lashbrooke *et al.*, 2016).

One recently elucidated MYB-type transcription factor (TF), SUBERMAN (SUB) or MYB39, in *A. thaliana* works to transactivate promoters of several suberin-associated genes, to control the establishment of endodermal root suberin lamellae (Cohen *et al.*, 2020). Similarly, in other studies of *A. thaliana*, the Rowland lab found that AtMYB41 is capable of activating the synthesis, export, assembly, and cell wall-localized deposition of suberin and suberin-associated waxes (Kosma *et al.*, 2014). Overexpression of *AtMYB41* greatly upregulates suberin biosynthetic gene expression, and results in production of suberin lamellae in leaves. AtMYB41 is not expressed in roots during regular plant development, but instead is induced in the root endodermis by abiotic stresses (Kosma *et al.*, 2014). Thus, AtMYB41 appears to be a key regulator of suberin in roots under stress conditions.

MdMYB93 from russeted apple (*M. domestica*) varieties, which is the predicted ortholog of AtMYB93, is able to induce suberin biosynthesis and deposition upon transient expression in *Nicotiana benthamiana* leaves (Legay *et al.*, 2016). It was shown by RNA-Seq analysis that MdMYB93 up-regulated genes involved in lipid, phenylpropanoid, and cell wall metabolism, as well as genes encoding for ABCG family transporters, most of which are predicted to be involved in the suberin biosynthesis pathway. Gas chromatography coupled with mass spectrometry (GC-MS) was used to investigate changes in leaf suberin composition.

The results demonstrate the role of MdMYB93 as a key regulator in the suberin pathway, involving biosynthesis of precursors, transport out of the cytosol, and assembly of the polymerized suberin structure in russeted apple fruits (Legay *et al.*, 2016).

Researchers have also reported that transient overexpression of AtMYB92 in *Nicotiana benthamiana* leaves up-regulates expression of aliphatic suberin biosynthetic genes, demonstrated by a 50-fold increase in suberin deposition and an overall increase in transcript levels of suberin biosynthetic genes (To *et al.*, 2020). Through promoter binding and promoter expression experiments, it was discovered that MYB92 binds to two adjacent MYB *cis*-elements to activate the promoter of a gene encoding for a subunit protein of the acetyl-coA carboxylase (ACCase) enzyme (BIOTIN CARBOXYL CARRIER PROTEIN2, BCCP2). In fact, all subgroup S24 MYB TFs (MYB53/92/93) were shown to activate *BCCP2* gene expression. ACCase is essential to the fatty acid biosynthesis pathway, which is upstream of suberin monomer synthesis (To *et al.*, 2020).

In the Rowland lab, much work has been done to characterize additional MYBs involved in regulation of the suberin pathway. It was discovered that AtMYB53, AtMYB92, and AtMYB93 have overlapping functions in the positive transcriptional regulation of suberin biosynthesis in young roots during development of *A. thaliana* under non-stress conditions (Hu, 2018). Similar to other MYBs, it is possible that expression of these TFs are also induced by stress, such as drought and salinity. It is now known that AtMYB53, AtMYB92 and AtMYB93 can be individually overexpressed to increase suberin production (Murmu *et al.*,

manuscript in preparation) (Hu, 2018). From studies of *myb53*, *myb92* and *myb93* loss-of-function single, double and triple mutants, it was shown that mutant plants have decreased suberin levels compared to wild-type (WT). Single gene knock out (KO) mutants of these genes each had reduced total suberin compared to WT (19-29%), double mutants had further decreased suberin (39-57%), and triple KO had the lowest suberin levels compared to WT (72%). These results indicate that AtMYB53, AtMYB92 and AtMYB93 have individual and overlapping functions in the positive regulation of the suberization process (Hu, 2018). Despite this knowledge, more studies are required to further elucidate the molecular mechanisms by which these MYB TFs regulate gene expression associated with suberin deposition.

1.5. MYB protein-protein interactions

Much like most other transcription factors, MYB-type TFs require other proteins (i.e. binding partners) to form complexes to bind to promoter sites and regulate expression of the cognate gene. These protein-protein interactions (PPIs) are of great importance to the elucidation of biological processes, but not much work has been done to identify MYB PPIs in relation to the transcriptional regulation of suberin-related genes. Despite the lack of knowledge in this subject, it is known that MYB TFs typically interact with basic helix-loop-helix (bHLH) TFs as part of larger protein complexes when bound to the promoters of target genes in plants (Feller *et al.*, 2011).

The plant bHLH TF protein family is functionally diverse, consisting of over two-dozen subfamilies, responsible for regulating transcription of genes involved in

many essential developmental and physiological roles. Plant bHLH TFs can act as transcriptional activators or repressors, depending on the regulatory pathway (Li *et al.*, 2006) (Feller *et al.*, 2011). A schematic structure of a typical bHLH protein is shown in Figure 1.9. The conserved C-terminal DNA BD consists of a bHLH domain, which is broken down into leucine zipper basic amino acid region (b) and a helix-loop-helix motif (HLH). The former is involved in DNA binding to E-box (5'-CANNTG) and G-box (5'-CACGTG) sequences with a highly conserved HER motif (His5-Glu9-Arg13), while the latter consists of two amphiphathic α -helices joined by a loop region of variable length (Feller *et al.*, 2011) (Pireyre and Burow, 2015). The HLH motif typically forms dimers with other bHLH proteins, or in some cases with non-bHLH proteins, such as R-interacting factor 1 (RIF1), an AGENET domain-containing EMSY-like protein (Hernandez *et al.*, 2007). This dimerization is required prior to DNA binding and contributes to DNA binding sequence specificity (Feller *et al.*, 2011).

Figure 1.9: Schematic diagram of plant bHLH proteins. The N-terminal transactivation domain (TAD) is upstream of a C-terminal multi-feature DNA binding domain (DBD). This DBD contains a nuclear localization sequence (NLS), dimerization domain (DD), basic helix-loop-helix structure (bHLH), and a DNA binding motif (E/G-box). Monomeric bHLH proteins will often dimerize before DNA recognition is initiated (Feller *et al.*, 2011) (Pireyre and Burow, 2015).

Studies regarding the PPI specificities of plant MYB proteins have discovered a conserved amino acid sequence for interaction with bHLH proteins; the motif [DE]Lx₂[RK]x₃Lx₆Lx₃R at position 12-33 in the R3 domain is shared among 14 R2R3 MYBs that interact with R/B-like bHLHs (Zimmermann *et al.*, 2004)(Zhao *et al.*, 2013). Site-directed mutagenesis complimented by yeast-two hybrid (Y2H) experiments demonstrated that any change to the amino acid sequence, located on helices 1 and 2 of the R3 repeat, results in loss of interaction (Zimmermann *et al.*, 2004). Specifically, two amino acids (L20, R33) are responsible for the majority of interaction strength. This motif is so conserved across some MYBs that it can be used to predict MYB-bHLH PPIs *in silico* (Zimmermann *et al.*, 2004), but it cannot alone be used to explain the molecular interaction mechanisms of specific bHLH proteins. (Pireyre and Burow, 2015).

As an example, it was recently discovered that MYB1 and bHLH1/3 TFs interact to functionally regulate genes involved in anthocyanin biosynthesis during pigment accumulation in lychee fruit (*Litchi chinensis*) (Lai *et al.*, 2016). In another study, a genome-wide analysis study of bHLH TFs in foxtail millet (*Setaria italica*) was able to clarify the function of *bHLH* genes in this drought-tolerant crop. It was strongly inferred that several *SibHLH* TFs have a key role in drought tolerance, by regulating major drought-induced genes in foxtail millet (Wang *et al.*, 2018). It is possible that yet uncharacterized bHLHs function in a similar manner in the regulation of suberin-associated genes, by coordinating with MYB TFs to control suberin-related genes in response to drought stress.

Cytosolic repeat proteins (WDR), such as WD40-repeat motif proteins, often associate with MYBs and bHLHs to form trimeric complexes of MYB/bHLH/WDR (MBW) before downstream transcription can be initiated (Feller *et al.*, 2011). The WD40-repeat motif is named according to the conserved Trp-Asp (WD) dipeptide with ~40 amino acid residues in a single repeat. WD40-repeat proteins serve largely as interaction platforms for PPIs and protein-DNA interactions of several cellular processes, such as signal transduction, vesicular trafficking, and transcriptional regulation (Xu and Min, 2011). Each WD40 repeat consists of a four-stranded antiparallel β -sheet, forming a strong hydrogen bond network to stabilize the folds. This stable structure of the WD40 domain, with its insertion and extension motifs, acts as a rigid scaffold for PPI and protein-DNA complexes to form. In addition, the domain has been shown to coordinate downstream events, such as PTMs (Xu and Min, 2011).

MBW complexes exhibit transcriptional regulation of multiple cellular differentiation pathways in plants (Ramsay and Glover, 2005). With reference to Table 1.1, experiments involving Y2H and Y3H assays have shown that MBW complexes function as transcriptional regulators of epidermal cell differentiation of trichomes and root hairs (Pesch *et al.*, 2015), as well as plant defense pathways such as anthocyanin biosynthesis (Zhang *et al.*, 2003). In addition, certain AtMYB/MYC/WD40 complexes appear to be involved in positive regulation of trichome and root hair patterning, revealed by Y2H and bi-molecular fluorescence complementation (BiFC) assays (Zhao *et al.*, 2012). MYB/bHLH/TCP complexes are also involved in plant defense response, promoting flavonoid biosynthesis and auxin

response. TCP proteins belong to the bHLH TF family in plants, and TCP3 has been shown to interact in a trimeric complex with R2R3-MYB and TT8 (bHLH) to strengthen transcriptional activation. These PPIs were determined by Y2H, Y3H, and BiFC assays. (Li and Zachgo, 2013). As an example of negative regulation, physical PPIs of SPL TFs with MYBs work to disrupt MBW complexes involved in anthocyanin biosynthesis, as demonstrated by Y2H, PCA, and co-immunoprecipitation (Co-IP) (Gou *et al.*, 2011). The *SPL* gene family encode a group of functionally diverse TFs exclusive to plants, and are identified by a conserved SBP DNA BD that is 76 amino acids in length (Cardon *et al.*, 1999). Other stress-responsive pathways regulated by MYB complexes include biosynthesis of phytohormones (e.g. giberellic and jasmonic acids) (Feller *et al.*, 2011).

MBW and other TF complexes are regulated by PTMs, such as ubiquitination, lysine acetylation, phosphorylation, redox, sumoylation, and proteolytic cleavage, likely at TAD sites of complexed proteins (Pireyre and Burow, 2015), possibly mediated by WD40-repeat proteins. Recruitment of TFs to promoter sites can be determined by both PTMs and PPIs. Thus, interaction of MYBs with certain proteins may direct the MYBs to promoters of suberin biosynthetic genes in the nucleus. The study of these PPIs is necessary, as it will reveal important aspects of AtMYB function in relation to suberin biosynthesis gene regulation.

AtMYB TFs do not always interact as MBW complexes. MYBs can interact with other proteins in the absence of bHLH and WD40. MYB46 is a specialized TF that functions as the master regulator for genes involved in biosynthesis of all three major components of secondary cell walls (cellulose, hemicellulose, and lignin), as

well as other downstream TFs in this pathway (Ko *et al.*, 2014). Secondary cell walls are responsible for the majority of plant biomass. They are formed in xylem cells for mechanical strength and water movement through stems, and in seed coats for protection. MYB46 and MYB83 transcriptionally regulate secondary cell wall biosynthesis genes. MYB46/83 are thought to directly regulate gene expression of downstream MYB43/58/63, and function cooperatively with MYB4/7/32/52/54 TFs (Ko *et al.*, 2014). In addition to downstream MYBs, MYB46/83 also regulate expression of KNAT7 TF (knotted-like homeobox, KNOX). KNAT7 has been shown to form PPIs with MYB75 (Table 1.1), and models have been devised where MYB75/KNAT7 form a trimer with an OFP (ovate family protein) to inhibit expression of lignin biosynthetic genes in *Arabidopsis* inflorescence stem. It is important to note that this complex does not appear to involve bHLH or WD40 proteins. Alternatively, MYB75/KNAT7 has been proposed to form a tetrameric complex with MYB5 and bHLH42 (TT8, transparent testa 8) to inhibit secondary cell wall genes in seed coats of *Arabidopsis* (Bhargava *et al.*, 2013), which does indeed involve a bHLH TF, but still no WD40-repeat protein. Through the formation of different complexes, regulatory activity can be organized in a function-specific and tissue-specific manner. These are related examples of MYBs acting as master regulators for downstream biosynthetic genes and TFs, providing evidence that MYBs are able to interact and function with other proteins, without the need to form a MBW trimeric complex. For this reason, future work with MYBs should involve discovering novel PPIs and how their diversity relates to their intricate regulatory abilities of biological processes.

Table 1.1: Examples of experimentally proven PPIs for AtMYB TFs and their biological function. Table was adapted from Bemer *et al.* (2017)

Protein families	Specific members	Function(s)	Identification method(s)	Reference
MYB bHLH WD40 <i>MBW complex</i>	GL1 GL3 WD40	Epidermal cell differentiation of trichomes and root hairs	-Y3H -Pull-down assay with LUMIER (luminescence-based interactome assay) -FRET (<i>Nicotiana benthamiana</i>)	(Pesch <i>et al.</i> , 2015)
MYB bHLH WD40 <i>MBW complex</i>	MYB75 GL3 WD40	Anthocyanin pigment biosynthesis	-Y2H	(Zhang <i>et al.</i> , 2003)
MYB MYC WD40	AtMYB AtMYC1 WD40	Positive regulation of trichome and root hair patterning	-Y2H -BiFC	(Zhao <i>et al.</i> , 2012)
MYB SPL	R2R3-MYB SPL9	Possibly disrupts the MBW complex involved in flavonoid biosynthesis	-Y2H -PCA (protein complementation assay) -Co-IP (<i>N. benthamiana</i>)	(Gou <i>et al.</i> , 2011)
MYB bHLH TCP	R2R3-MYB TT8 TCP3	Plant defense response, promoting flavonoid biosynthesis, auxin response	-Y2H -Y3H -BiFC -GUS assay (for co-expression)	(Li and Zachgo, 2013)
MYB KNOX	MYB75 KNAT7	Negative regulation of lignin biosynthetic genes in stems, and of secondary cell wall genes in seed coats	-Y2H -BiFC -GUS assay (for co-expression)	(Bhargava <i>et al.</i> , 2013)

1.6. Thesis project rationale & objectives

Suberin has been an increasingly important topic of plant research over the past 15 years, notably in the model organism *A. thaliana*, and in the crops *S. tuberosum* (potato) and *Quercus suber* (cork oak). Considerable work has been done to elucidate the suberin biosynthesis pathway (Vishwanath *et al.*, 2015), but still much of the underlying mechanisms governing regulation of suberin deposition are yet to be discovered.

As presented in Chapter 1.4, my thesis project expands on these findings, building on recent transcriptomic analysis in *Arabidopsis* (performed by Daniel Klein, Rowland Lab) using a steroid-inducible *AtMYB53* overexpression (OE) line and two *myb53 myb92 myb93* triple knockout mutant lines that are affected in suberin deposition (Hu, 2018). Specifically, Klein (2019) used RNA-Seq analysis to compare the root transcriptomic profiles of the two *myb53 myb92 myb93* triple knock-out lines with that of wild-type plants (plants were 14-days old and grown in tissue culture). Secondly, the steroid-inducible *AtMYB53-OE* line was grown for 10 days on tissue culture media, and then overexpression of *AtMYB53* induced using the steroid β -estradiol for 6 hours (early time point) or 15 hours (late time point). The transcriptomic profiles, measured by RNA-Seq, of both time points were compared between the steroid-induced and control (no steroid added) plants. The combined datasets generated valuable new information about the overall regulation and production of suberin, as governed by the *AtMYB53*, *AtMYB92* and *AtMYB93* transcription factors. For example, from Klein's RNA-Seq data, *bHLH27*, *bHLH112* and two genes encoding WD40-repeat proteins were differentially regulated by

MYB53/MYB92/MYB93 in root tissue, meaning that these candidate genes are likely downstream targets of these AtMYBs (Klein, 2019).

From these transcriptomic data, I hypothesized that bHLH proteins function in a feedback regulation manner with the MYB TFs (Figure 1.10), where downstream bHLH genes are regulated by MYBs, and these bHLH proteins then form PPIs with these same MYBs to regulate themselves and other genes, such as suberin biosynthetic genes. Precedent for this model comes from previous literature on *Arabidopsis* (Baudry *et al.*, 2006) and poplar MYBs (James *et al.*, 2017). I also hypothesized that WD40-repeat proteins would be important for the bHLH-MYB interaction (MBW complex), and thus affect expression of downstream suberin biosynthetic gene expression.

Figure 1.10: Feedback regulation model of MYB/bHLH PPI for suberin biosynthetic genes. WD40 scaffolding proteins may also be involved, since MBW complexes are common for regulatory complexes in plants (not shown). Precedent for this model comes from recent literature on *Arabidopsis* and poplar MYBs (Baudry *et al.*, 2006)(James *et al.*, 2017).

The study of TF complexes involving suberin-associated AtMYBs will provide invaluable insights to our mechanistic understanding of transcriptional regulation of suberin-associated genes. The major goal of this thesis was to determine how AtMYB53, AtMYB92, and AtMYB93 activate the expression of different genes depending on their specific PPIs with other proteins (AtbHLHs and AtWD40s). Several approaches were taken in my MSc studies to obtain this information, and several hypotheses were devised (Figure 1.11).

Figure 1.11: Summary of thesis rationale and objectives. Transcription factors AtMYB53, AtMYB92, and AtMYB93 have been shown to positively affect suberin content in root endodermis (Murmu *et al.*, manuscript in preparation) (Hu, 2018) (Klein, 2019). There is evidence that these MYBs regulate the expression of bHLH proteins (Klein, 2019), and they potentially interact directly with each other. In addition, WD40-repeat scaffolding proteins are involved in the interaction of several trimeric protein complexes, so a protein interaction model for this MBW relationship was also investigated. The expression patterns of these bHLHs were studied using promoter : GUS reporter analyses. In addition, the effects of suberin production in gene knock-outs were tested by quantitative chemical analysis of suberin monomer content in roots of young seedlings.

CHAPTER 2: MATERIALS AND METHODS

2.1: Yeast two-hybrid assays for detecting binary PPIs

All Y2H mating and cDNA library screening procedures were carried out following the Matchmaker® Gold Yeast Two-Hybrid System (630489, Takara Bio). All cDNA bacterial cultures were ordered from ABRC, excluding bHLH111 (Appendix, Table A1). These were grown overnight in liquid broth media and plasmids were extracted using the Presto™ Mini Plasmid Kit (PDH300, Geneaid). The coding sequences (CDS) of cDNA plasmids were amplified using PCR primers (Appendix, Table A5) with high-fidelity iProof™ Taq polymerase (172-5331, BioRad). MYB N-terminal CDS transcripts (750bp) were obtained using PCR primers (Appendix, Table A5) with high-fidelity iProof™ Taq polymerase (172-5331, BioRad). WD40 prey (pGADT7, *EcoRI*+*Bam*HI) and bHLH prey (pGADT7, *NdeI*+*XhoI*), along with MYB bait (pGBKT7, *Bam*HI+*Sall*) and bHLH bait (pGBKT7, *NdeI*+*Sall*) vector constructs were created using In-Fusion® HD Cloning (121416, Takara Bio). Constructs were transformed into chemically competent Stellar™ (F-, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ 80d lacZΔ M15, Δ(lacZYA-argF) U169, Δ(mrr-hsdRMS-mcrBC), ΔmcrA, λ-) or DH5α (*fhuA2 lac(del)U169 phoA glnV44 Φ 80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*) *E. coli*. The inserted coding sequences were verified by DNA sequencing (Eurofins Scientific, Toronto). These CDS fusion protein constructs were tested using Y2H for binary PPIs in yeast (*S. cerevisiae*; strain Y2H Gold™ [*MATa*, *trp1-901*, *leu2-3, 112*, *ura3-52*, *his3-200*, *gal4Δ*, *gal80Δ*, *LYS2:: GAL1_{UAS}-Gal1_{TATA}-His3*, *GAL2_{UAS}-Gal2_{TATA}-Ade2*, *URA3:: MEL1_{UAS}-Mel1_{TATA}, AUR1-C MEL1*]) with the following combinations:

MYB baits + bHLH preys; MYB baits + WD40 prey; bHLH baits + WD40 prey. All (co-)transformations were performed according to the small-scale LiAc yeast transformation procedure from Clontech's "Yeast Protocols Handbook". All combinations of MYB baits + bHLH preys and MYB baits + WD40 prey were tested for protein-protein interactions (Chapter 2.4).

2.2: bHLH111 deletion constructs for use in yeast two-hybrid assays

Regions of the coding sequence of *bHLH111* on a plasmid template (from stock OR 1762) were amplified using various PCR primer combinations (Appendix, Table A5) with high-fidelity iProof™ Taq polymerase (172-5331, BioRad) to create two separate protein deletion products: N-bHLH111 [HLH region deletion, 324-364 aa] and C-bHLH111 [basic region deletion, 307-323 aa]. These internal deletions were created using overlap PCR designed with In-Fusion® overhang regions. Deletion CDS regions, N-bHLH111 and C-bHLH111 prey (pGADT7, *NdeI*+*XhoI*), were cloned by In-Fusion® HD Cloning (121416, Takara Bio). The inserted coding sequences were verified by DNA sequencing (Eurofins Scientific, Toronto). These protein deletion constructs were transformed into Stellar™ or DH5α *E. coli*, followed by (co)-transformation into Y2H Gold™ yeast (following Chapter 2.1), and tested for MYB bait + bHLH deletion PPIs (Chapter 2.4), to confirm positive result from Y2H studies.

2.3: Yeast three-hybrid assays for detecting trimeric protein complexes

The coding sequences of Y3H bait and prey constructs were amplified using PCR primers (Appendix, Table A6) with high-fidelity iProof™ Taq polymerase (172-5331, BioRad). MYB bait (pBridge MCSI, *EcoRI*+*SaII*) and WD40 bridge CDS (pBridge MCSII, *NotI*+*BglII*) were cloned by In-Fusion® HD Cloning (121416, Takara Bio) into the same pBridge vector (630404, Takara Bio), creating MYB92/WD40 and MYB93/WD40 bait constructs. The inserted coding sequences were verified by DNA sequencing (Eurofins Scientific, Toronto). These CDS fusion protein constructs were transformed into Stellar™ or DH5α *E. coli*, followed by (co)-transformation into Y2H Gold™ yeast (following Chapter 2.1), and tested for MYB/WD40 baits + bHLH preys protein-protein interactions (Chapter 2.4).

2.4: Drop-inoculation plating for yeast two/three-hybrid (co)-transformants

Overnight cultures of tested yeast (co)-transformants were prepared in 5 mL of selective liquid media (SDO [-Leu] or DDO [-Trp/-Leu] or TDO [-Trp/-Leu/-Met]), incubated for 16-20 hours at 30°C with shaking (280 RPM). Growth of cultures was quantified before use ($OD_{600} \sim 1.0-1.5$). Under sterile conditions, 1 mL of culture was centrifuged at $1200 \times g$ for 10 min at room temp. Supernatant was discarded and pellet was resuspended in dH₂O to obtain $OD_{600} \sim 6.0$ in the stock solution. A 10X dilution series was created to obtain 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} solutions, and 2 µL of each dilution was plated in a row on control plates (SDO [-Leu] or DDO [-Trp/-Leu] or TDO [-Trp/-Leu/-Met]) and treatment plates (QDO [-Trp/-Leu/-Ade/-His] or PDO [-Trp/-Leu/-Met/-Ade/-His]). Plates were incubated at 30°C for 2 days.

The recipe for synthetic drop out (SD) media consists of: yeast nitrogen base without amino acids ($6.7 \text{ g}\cdot\text{L}^{-1}$), agar ($20 \text{ g}\cdot\text{L}^{-1}$), D-glucose (2%), and L-amino acid/nucleotide mix ($30 \text{ mg}\cdot\text{L}^{-1}$ Ile, $150 \text{ mg}\cdot\text{L}^{-1}$ Val, $20 \text{ mg}\cdot\text{L}^{-1}$ Arg, $30 \text{ mg}\cdot\text{L}^{-1}$ Lys, $50 \text{ mg}\cdot\text{L}^{-1}$ Phe, $200 \text{ mg}\cdot\text{L}^{-1}$ Thr, $30 \text{ mg}\cdot\text{L}^{-1}$ Tyr, $20 \text{ mg}\cdot\text{L}^{-1}$ Uracil), with final pH of 5.8 before autoclaving. Other supplementary L-amino acids/nucleotides were added in varying combinations to achieve different drop-out recipes: $20 \text{ mg}\cdot\text{L}^{-1}$ Met, $100 \text{ mg}\cdot\text{L}^{-1}$ Leu, $20 \text{ mg}\cdot\text{L}^{-1}$ Trp, $20 \text{ mg}\cdot\text{L}^{-1}$ His, $100 \text{ mg}\cdot\text{L}^{-1}$ Ade.

2.5: Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. plants used in these studies were of the Columbia (Col-0) ecotype background. The T-DNA insertion lines for mutant plants (Appendix, Table A1) were obtained from the SALK institute T-DNA Express website (signal.salk.edu/cgi-bin/tdnaexpress), and ordered from the Arabidopsis Biological Resource Centre, ABRC (arabidopsis.org).

All *A. thaliana* seeds (WT and T-DNA mutants) were surface-sterilized by washing with 70% ethanol for 2-3 min, followed by immersion in 1.5% (v/v) sodium hypochlorite plus 5% (v/v) sodium dodecyl sulfate (SDS, L4390, Sigma) for 2 min. Sterilized seeds were washed and rinsed 6 times with sterile distilled water, then stratified for 3-4 days at 4°C in the dark. Plants were grown on $\frac{1}{2}\text{MS}$ + sucrose media ($2.22 \text{ g}\cdot\text{L}^{-1}$ Murashige and Skoog (MS) basal medium with Gamborg vitamins [M404, PhytoTech Labs], $50 \text{ mg}\cdot\text{L}^{-1}$ MES buffer, $10 \text{ g}\cdot\text{L}^{-1}$ sucrose, 0.525% agar, pH 5.7-5.8) plates, or in sterilized potting soil (BX-General Purpose growing medium, 10380RG, Pro-Mix) fertilized with $1 \text{ g}\cdot\text{L}^{-1}$ fertilizer (20(N)-20(P)-20(K) Classic,

10529, Plant-Prod) plus 0.0625 g·L⁻¹ micronutrients (chelated micronutrient mix, 10046, Plant-Prod). All plants for were grown in soil from seeds in an environmental growth chamber at 22-23°C with 30-60% humidity, a 16h/8h light/dark cycle, and a light intensity of 80-150 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

2.6: *bHLH111* promoter : GUS reporter assays

DNA fragment (2660 bp) representing the upstream region up to the start codon of the *bHLH111* gene ('promoter') was obtained from WT *A. thaliana* genomic DNA using PCR primers (Appendix, Table A4) with high-fidelity iProof™ Taq polymerase (172-5331, BioRad). The *bHLH111* promoter vector construct (Promoter_{bHLH111}:GUS) was created using TOPO-D pENTR system (K240020, ThermoFisher) with the pKGWFS7.0 destination vector (ABRC). The construct was transformed into chemically competent TOP10™ (F- *mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *araD139* Δ (*ara-leu*)7697 *galU* *galK* *rpsL* (StrR) *endA1* *nupG*) *E. coli*. The inserted promoter fragment was verified by DNA sequencing (Eurofins Scientific, Toronto). The Promoter_{bHLH111}:GUS construct was transformed into electro-competent GV3101 *Agrobacterium tumefaciens* (C58 – *rif^R*– *Ti* *pMP90*–*pTiC58DT-DNA* – *gentR* –*Nopaline*). Transformed *A. tumefaciens* were used for floral dipping of *A. thaliana* (Clough and Bent, 1998) (Zhang *et al.*, 2006). Harvested seeds were plated on ½MS + sucrose + kanamycin (50 mg·L⁻¹) media and grown under 24h light. Viable transgenic seedlings (T₁ generation) were transplanted to soil, and were grown until harvest of T₂ seeds.

12-day-old seedlings from 15 independent lines of T₂ generation were used as samples for GUS-staining, with full length (2300 bp) FAR4 promoter:GUS T2 seedlings as positive control (C+) and WT (Col-0) seedlings as negative control (C-). All seedlings were grown on ½MS + sucrose plates, 24h light. GUS-staining solution was prepared by dissolving 2.5 mg X-Gluc (G1281C1, GoldBio) in 50 µL DMF (227056, Sigma) then adding to a solution of 50mM NaPO₄ buffer + 4mM KFe(CN)₆ + one drop of Triton X-100 (TRX777, BioShop) with gentle inversion to mix. In 5 mL conical tubes, ten seedlings of each sample were immersed in 2 mL of GUS-staining solution. After 4 hours at 37°C in the dark, half of the stained seedlings were transferred to 70% (v/v) ethanol in clean scintillation vials. The remaining seedlings were left to stain at 37°C in the dark overnight (22 hours), then transferred to 70% ethanol in clean scintillation vials, separate from 4-hour-stained seedlings. Multiple staining time points were used to achieve optimal staining levels, due to differences in promoter expression across lines. The following day, seedlings were immersed in fresh 70% ethanol, and samples were stored until observed using bright-field light microscopy with stereomicroscope (Zeiss Discovery V-20 SteREO microscope with PlanApo S 0.3X FWD 253mm lens) and compound microscope (Zeiss Axio Imager.M2 with Plan-APOCHROMAT 10X, 20X objectives) using AxioVision 4.8 software.

2.7: Identification of homozygous T-DNA insertion lines

PCR-based genotyping was used to identify plants homozygous for the T-DNA insertion (example in Chapter 3.4, Figure 3.4.2). Genomic DNA was isolated from leaf tissue of 2 to 4-week-old soil-grown plants, using a standardized extraction method (Edwards *et al.*, 1991). Excised leaves were frozen at -20°C, and ground until well crushed in a microcentrifuge tube. To each sample, 400 µL of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% SDS) was added and mixed well. Each sample was centrifuged for 10 min at 21,000 $\times g$, then 350 µL of supernatant was immediately transferred to a new tube. To each tube, 350 µL of isopropanol was added and inverted gently to mix well and precipitate genomic DNA. Each sample was centrifuged for 10 min 21,000 $\times g$ and supernatant was discarded. To remove contaminant salts, the clear DNA pellet was resuspended in 800 µL of 70% ethanol, then centrifuged for 5 min at 21,000 $\times g$ and supernatant was discarded. The tubes were left to dry completely before resuspending the genomic DNA in 100 µL of dH₂O, then stored at 4°C or -20°C before use as template in PCR genotyping reaction.

Genomic DNA were genotyped by standard PCR reactions (a. 95°C for 5 min, b. 95°C for 30 sec, c. 55°C for 30 sec, d. 72°C for 1 min per 1000 bp product, e. repeat steps b-d for 40 cycles total, f. 72°C for 5 min) with gene specific and T-DNA specific primer pairs Appendix, Tables A2,A3). Seeds from individual homozygous mutant plants were harvested and were further propagated for downstream analysis of suberin-associated phenotypes.

2.8: Chemical analysis of root suberin using gas chromatography

Seeds for suberin analysis were sown on $\frac{1}{2}$ MS + sucrose plates and grown under continuous light (22-23°C, 30-60% humidity, $100\text{-}150\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), using 60 seedlings per plate, four plates per replicate, and four technical replicates per mutant line (~95% germination rate). Root tissue from 14-day-old seedlings was harvested (using chloroform-rinsed tweezers and razor blades) for quantitative characterization of lipid polyester composition (suberin) in the T-DNA loss-of-function mutant lines compared to WT *A. thaliana*. Lines chosen for analysis: bHLH27 (SAIL_104_C12), bHLH111 (SALK_001970), bHLH112 (SALK_148540), WD40 (DWA3) (SALK_046984), and WD40 (SAIL_255_C01).

Tissue delipidation (removal of non-polymerized lipids) and base-catalyzed de- polymerization of lipid polyesters (suberin) were carried out prior to suberin analysis by gas chromatography. To avoid any carry-over of external lipid contamination, all glassware was cleaned three times with chloroform (reagent grade x2, HPLC grade x1), and all glass tube caps were rinsed three times with 50% (v/v) methanol. For delipidation, all samples of root tissue were rapidly collected after being chopped into a paste and immersed in hot isopropanol (pre-heated in an 85°C oven) for 15 min at 85°C. After cooling to room temperature, broken tissue was mechanically homogenized using a small hand-held electric polytron (also rinsed three times with chloroform). Soluble lipids were removed from the ground samples (delipidation) by incubating with a series of mixed solvents of increasing polarity: 2:1 (v/v) chloroform : methanol, 1:1 (v/v) chloroform : methanol, (1:2) (v/v) chloroform : methanol, and 100% methanol, successively (chloroform, HPLC

grade, CA71006- 912, Anachemia; methanol, HPLC grade, CA11020-604, Anachemia). Each solvent extraction step of the series lasted for 24-72 h, including changing the solvent once during each step. All samples were inverted on a tilting table during the delipidation process to ensure thorough mixing.

Following delipidation, all samples were dried in a fume hood at room temperature for several days and then transferred to a sealed desiccator with silica gel beads for at least a few days. The resulting dried residues were weighed and recorded. Ten μg of each internal standard, heptadecanoic acid (H3500, Sigma) and ω -pentadecalactone (W284009, Sigma), were added to each sample replicate. The lipid polyesters were depolymerized by base-catalyzed trans-esterification at 60°C for 2 h by adding 0.9 mL methanol, 0.225 mL methyl acetate (296996, Sigma), and 0.375 mL sodium methoxide (156256, Sigma) to each tube. Samples were vortexed thoroughly every 30 min during the reaction. After cooling down, 1 mL of glacial acetic acid (00598-463, Anachemia) was added to stop the reaction to adjust the pH to 4-5. Then, 2.5 mL of dichloromethane (32667-360, Anachemia) and 2 mL of 0.5 M NaCl was added to each sample to extract the depolymerized monomeric constituents. The mixtures were vortexed thoroughly and centrifuged for 5 min at 805 x *g*. The compounds were obtained in the organic phase, which was then washed three times with 1 mL of 0.5 M NaCl, extracting into the organic phase each time. Anhydrous sodium sulfate (SOS513, BioShop) (around one-half volume of the organic phase) was added, and left overnight to remove residual water from the organic phase.

The following day, samples were centrifuged at 805 $\times g$ and the anhydrous organic phase transferred to clean tubes. Extracts were placed under a gentle stream of nitrogen gas at 37°C until evaporated. The free hydroxyl groups were then acetylated (derivatized) using 100 μL acetic anhydride (351001, Fisher) + 100 μL pyridine (270970, Sigma) at 60°C for 2 hours, vortexing occasionally. The samples were again evaporated under a gentle stream of nitrogen at 37°C. Dried samples were then re-dissolved in 50 μL heptane (270512, Sigma) + 50 μL toluene (270377, Sigma) for analysis by gas chromatography.

An Agilent 7820A gas chromatograph equipped with a FID (flame ionization detector) was used for analysis. One μL of each sample was injected using a glass 10 μL Hamilton syringe fitted onto an autosampler. The samples were injected into a splitless injector at 300°C. Individual monomers were separated on an Agilent HP-5MS capillary column (30 m length, 0.250 mm diameter, 0.25 μm film thickness). The oven temperature program was as follows: initial temperature of 140°C for 3 min, then ramped up 10°C/min until 310°C and held for 17 min (37 min run time per sample). The monomers were detected by FID set at 350°C. Helium was used as carrier gas at a constant flow of 1.5 mL/min, nitrogen (28 mL/min) was used as make-up gas, while air (300 mL/min) and hydrogen (30 mL/min) were used as combustion gases. Peak identification was done by comparison with retention time patterns of samples that were characterized by gas chromatography-mass spectrometry (GC-MS).

2.9: Quantification of suberin monomers using flame ionization detection

The FID response is proportional to the carbon atom number and the degree of unsaturation of the analytes. It was not feasible to generate external calibration curves for all the different suberin monomeric constituents. Therefore, to calculate these variant FID responses to individual components, correction factors are used to amend the peak areas of the signal produced by individual suberin monomers and then detected by FID (Mossoba *et al.*, 2014). Calculations were performed using an Excel spreadsheet, modified from an original template (Jenkin and Molina, 2015).

$$\text{Corrected peak area} = \frac{\text{relative peak area (pA * s)}}{\text{FID correction factor}}$$

The corrected peak areas of the suberin monomers were then compared to the corrected peak areas of C17:0 fatty acid and C15:0 ω -hydroxy fatty acid (linearized product of ω -pentadecalactone) as the two internal standards (IST). For both of these two internal standards, 10 μg was added to each sample to determine the amount of each suberin monomer, which was represented as the $m_{(\text{suberin monomers})}$:

$$m_{(\text{suberin monomer})} = \frac{\text{corrected peak area}_{(\text{suberin monomer})}}{\text{corrected peak area}_{(\text{IST})} \times 10 \mu\text{g}}$$

Since all monomers were acetylated, $m_{(\text{suberin monomer})} = n_{(\text{acetylated suberin monomer})} \times \text{MW}_{(+\text{Ac})}$. Thus,

$$n_{(acetylated\ suberin\ monomer)} = \frac{n_{(acetylated\ suberin\ monomer)} \times MW_{(+Ac)}}{corrected\ peak\ area_{(IST)} \times 10\ \mu g}$$

The $n_{(acetylated\ suberin\ monomer)}$ was used to calculate the amount of suberin monomers as the underivatized suberin monomers in the original biopolymer, as $\mu g/mg$ of root dry weight (DW):

$$m_{(polymerized\ suberin\ monomer)} = n_{(acetylated\ suberin\ monomer)} \times MW_{(+Ac)}$$

2.10: Seed viability assays for identification of homozygous lethality in select T-DNA lines

In addition to PCR-based genotyping, another strategy to test embryo lethality / seed viability was through dissection of mature siliques (at both green and dried stages) to observe seed morphology and distribution. Images were captured using light microscopy (Zeiss Discovery V-20 SteREO microscope with PlanApo S 0.3X FWD 253mm lens) on AxioVision 4.8 software.

Seed viability ratios were quantified using a colourimetric test with tetrazolium red (TZ) salt (Verma and Majee, 2013). Under sterile conditions, about 100 seeds were submerged (in triplicate) in 1 mL of scarification solution (1.2% v/v sodium hypochlorite, 0.001% v/v Triton X-100) for 15 min under shaking conditions at room temperature. Heat-treated (100°C, 1h) WT seeds were used as negative control. Seeds were washed six times with dH₂O to completely remove bleach. Still under sterile conditions, excess water was removed and seeds

incubated in 1% TTC (2,3,5-triphenyl tetrazolium chloride) (17779-10, Sigma) solution at 30°C for 24 to 48 h in the dark. After staining, seeds were washed three times with dH₂O, then immersed in clearing agent (1:1:2:1, lactic acid: phenol : glycerol : water) for 2 h. Stained seeds were observed using a stereo-microscope and evaluated on the basis of staining pattern and colour intensity. Seeds with bright red staining were considered completely viable, partially stained seeds as abnormal, while pink or grayish red represented dead tissue and completely unstained seeds were non-viable. Images were captured using bright-field light microscopy (Zeiss Discovery V-20 SteREO microscope with PlanApo S 0.3X FWD 253mm lens) using AxioVision 4.8 software.

CHAPTER 3: RESULTS

3.1: Bioinformatics analysis of *AtbHLH27*, *AtbHLH111*, *AtbHLH112*, and the genes encoding proteins with WD40 motifs

In this study, I investigated the potential roles of five candidate genes in the regulated deposition of suberin in *Arabidopsis* root endodermis, three encoding bHLH TFs and two encoding WD40-repeat motif proteins. As described in Chapter 1.6, transcriptomic analysis by RNA-Seq of a steroid-inducible *AtMYB53* overexpression (OE) line along with two independent *myb53 myb92 myb93* loss-of-function (triple knockout, TKO) mutants revealed extensive alterations in RNA transcript levels in roots of young (10-14 days-old) *Arabidopsis* seedlings during development (Klein, 2019). Alterations included that of genes encoding suberin biosynthetic enzymes. From these data, genes encoding *AtbHLH27*, *AtbHLH112* and two WD40-repeat proteins were differentially regulated in the *AtMYB53* overexpression or *myb53 myb92 myb93* TKO lines (Table 3.1.1), meaning that these are downstream targets of the suberin-associated AtMYB TFs being studied here, either directly or indirectly, and may themselves be involved in the regulation of suberin deposition in root endodermis. The *bHLH112* gene increased expression levels 461-fold in the MYB53-OE line compared to control, six hours after *AtMYB53* overexpression using the steroid inducible line; this is much greater compared to the *bHLH27* gene, which experienced about a 4-fold increase six hours post-induction (Table 3.1.1). This strong up-regulation of the *bHLH112* gene makes it a candidate of special interest, likely being targeted by AtMYB53 for a specific reason, possibly related to suberin gene regulation. Another bHLH candidate, *AtbHLH111*,

was chosen to be part of this study because of its root co-expression with several suberin biosynthetic genes, such as *FAR4*, *FAR5*, *GPAT5*, *CYP86A1*, *CYP86B1*, and *ASFT* (Razeq, 2013). The gene of unknown function (*At3g50390*) encoding a protein with a WD40 motif was up-regulated in the TKO2 mutant line, with about 3.5-fold increase in expression level (Table 3.1.1). Six hours after *AtMYB53* overexpression using the steroid inducible line, *At3g50390* demonstrated an increase of transcript levels of approximately 3-fold, and fifteen hours post-induction, it had a 22 fold-increase in expression level compared to un-induced control. The other gene encoding a WD40-containing protein, *DWA3*, was up-regulated in the TKO1 mutant, with transcript levels almost 4-fold greater than wild-type (Table 3.1.1).

Phylogenetic analysis of a representative selection of AtbHLH TFs (Figure 3.1.1.) shows the distant relationship of AtbHLH27 (Group III) from AtbHLH111 and AtbHLH112 (Group X). AtbHLH111 is closely related to AtbHLH112, both with the same conserved domains in Group X (Heim *et al.*, 2003), and with protein sequence percentage identity of 46.6%, which suggests that they may be functionally similar proteins (Figure 3.1.1, Table 3.1.2). The gene structures of *AtbHLH111* and *AtbHLH112* are more similar to each other (46.6%) than *AtbHLH27* (111: 23.9%; 112: 34.2%). The former two genes have seven exons, and similar predicted peptide lengths of the encoded proteins, as opposed to the shorter *AtbHLH27* transcript with only 4 exons (Table 3.1.2).

Protein interaction analysis of AtbHLH27, AtbHLH111 and AtbHLH112 using STRING (string-db.org) did not identify any known suberin-associated proteins, but one notable result was a direct yeast 2-hybrid interaction between AtbHLH111 and

LSU1 (RESPONSE TO LOW SULFUR 1) (AT3G49580) (Figure S1). *LSU1* encodes a small protein (94 aa) that is localized to the nucleus with protein binding activity and responsive to sulfur starvation in roots, likely interacting with regulatory TF complexes. It has been shown that changes in suberization occur in response to sulfur deficiency (Barberon *et al.*, 2016). The *LSU1* promoter contains about 20 regulatory elements for TF binding, and a supposed coiled-coil type tertiary protein structure strongly suggestive of multimer formation (Sirko *et al.*, 2015). PPI analysis also revealed an interaction between bHLH27 and bHLH25 (At4g27850), and bHLH proteins are known to heterodimerize (Figure S1). The *bHLH25* gene is expressed in very low amounts within the root differentiation zone (Schmidt *et al.*, 2018), mostly in the vasculature and endodermis (Brady *et al.*, 2007) in regions of suberin deposition.

The WD40-repeat protein of unknown function, *At3g50390*, has not been previously studied, so very limited protein interaction and expression data are publically available for this gene. However, DWA3 is involved in protein complexes that act to negatively regulate ABA responses in several plant organs (Gaudinier *et al.*, 2018). Although it is not found in roots during normal development, the DWA3 protein is expressed in response to ABA signals or salt stress in roots, which may have implications for stress-related regulation of suberin deposition.

A highly useful online tool used for analysis is ProteomicsDB (proteomicsdb.org), which compiles both proteomic and transcriptomic experimental data, based on mass-spectrometry techniques and RNA-Seq, respectively (Schmidt *et al.*, 2018). The interactive database was created to

centralize protein expression data from the human genome, but has since been expanded into *Arabidopsis* experiments, where plant scientists can also benefit from the large compository of experimental protein information. *AtbHLH27* and *AtbHLH111* have relatively similar gene expression levels in young seedlings (Figure 3.1.2 A), with transcripts most abundant in the root differentiation zone, the general region where suberin is deposited. Expression levels are measured as the number of transcripts from a gene per million transcripts of total RNA (i.e. transcripts per million, TPM) in a given tissue. The root differentiation zone is comprised of many specific cell types, so the analyzed transcripts are not necessarily indicative of the specific region where suberin is deposited (i.e. endodermis). In the root differentiation zone, TPM levels are similar for both *AtbHLH111* (42.7 TPM) and *AtbHLH112* (41.5 TPM), but slightly lower for *AtbHLH27* (32.0 TPM). *DWA3* is even lower at 20.9 TPM, and only trace amounts of *At3g50390* transcripts (0.1 TPM) are found in the root differentiation zone (Figure 3.1.2 B). For comparison, *AtMYB93* has an expression value of 35.6 TPM in the root differentiation zone (data on *AtMYB39*, *AtMYB41*, *AtMYB53* and *AtMYB92* were not available through ProteomicsDB). The plant-wide expression of *AtbHLH112* has been demonstrated by semi-quantitative RT-PCR, located in root, rosette leaf, cauline leaf, flower, silique, and stem tissues (Wang *et al.*, 2014). In addition, *AtbHLH112* promoter : reporter assays show GUS activity in root vasculature, mainly the pericycle and stele, but possibly the endodermis as well (Wang *et al.*, 2014).

In addition, the BAR ePlant online tool (bar.utoronto.ca/eplant) was used to obtain tissue-specific expression information from microarray data (Brady *et al.*,

2007), where the relative expression level (REL) is representative of the relative level of mRNA transcript in a specific tissue, compared to the lowest detectable amount. It was found that the *AtbHLH111* and *AtbHLH112* genes have the greatest REL in xylem/phloem pericycle cells (111: 1579/1968; 112: 554/805), and phloem companion cells within the vasculature (111: 313; 112: 300) (Figure 3.1.3). The root endodermis displays intermediate expression, with *AtbHLH112* more than triple the levels of *AtbHLH111* (111: 52; 112: 163). Unfortunately, *AtbHLH27* and *AT3G50390* gene expression data were not available through the BAR tool, possibly because they were not included on the microarray chip. Other members of Group X (*AtbHLH103*, *AtbHLH114*, and *AtbHLH123*) (Figure 3.1.1) are also expressed in the root, but all of these have unknown functions as TFs. Interestingly, *AtbHLH103* and *AtbHLH114* have much higher REL in root endodermis (103: 642; 114: 2243) compared to *AtbHLH111* or *AtbHLH112*. Similar to *AtbHLH27*, tissue-specific expression data was not found for *AtbHLH123* on the BAR ePlant tool. Analysis of *DWA3* demonstrates the very low REL in all root cell types, the greatest being xylem pericycle (23) and procambium (14) (Figure 3.1.3).

Table 3.1.1: List of fold-change values (with standard error) for candidate genes examined in this study using transcriptomic data from previous RNA-Seq analysis of MYB *loss-of-function* mutants and steroid inducible *AtMYB53* overexpression line (Klein, 2019). TKO1, triple knockout 1 (*myb53-1 myb92-1 myb93-1*); TKO2, triple knockout 2 (*myb53-2 myb92-1 myb93-1*); OE, over-expression line (6 or 15 hours post-induction by β -estradiol steroid). Each line or timepoint was examined by RNA-Seq using two biological replicates, using RNA extracted from 14-day old roots (TKO) and 10-day old roots (AtMYB53-OE). Controls were wild-type Col-0 *A. thaliana* (TKO) and AtMYB53-OE with DMSO injection instead of steroid (AtMYB53-OE). Significant changes of at least two-fold are highlighted in red.

Name (with TAIR ID no.)	TKO1	TKO2	AtMYB53-OE (6h)	AtMYB53-OE (15h)
AtbHLH027 (AT4G29930)	0.65 ($\pm$ 1.12)	0.49 ($\pm$ 1.14)	3.63 ($\pm$ 1.10)	2.27 ($\pm$ 1.11)
AtbHLH112 (AT1G61660)	2.20 ($\pm$ 1.42)	1.06 ($\pm$ 4.77)	461 ($\pm$ 1.81)	0.83 ($\pm$ 7.60)
DWA3 (AT1G61210)	3.92 ($\pm$ 1.02)	1.82 ($\pm$ 1.52)	0.46 ($\pm$ 1.02)	1.47 ($\pm$ 1.08)
Unknown WD40 (AT1G50390)	1.36 ($\pm$ 2.45)	3.41 ($\pm$ 1.66)	3.14 ($\pm$ 1.33)	22.3 ($\pm$ 1.27)

Table 3.1.2: Selected attributes of AtbHLH27, AtbHLH111, and AtbHLH112

proteins. Phylogenetic group is assigned based on structural similarities, specifically the organization of conserved domains (Heim *et al*, 2003). Amino acid percent identity is from BLASTp multiple sequence alignment.

Name (with TAIR ID no.)	*Gene length (bp)	**CDS length (bp)	Peptide length (aa)	Exon no.	Phylogenetic group	Amino acid percent identity
AtbHLH027 (AT4G29930)	3386	792	264	4	III	27<->111 = <u>23.9%</u> 27<->112 = <u>34.2%</u>
AtbHLH111 (AT1G31050)	3892	1305	435	7	X	111 <-> 27 = <u>23.9%</u> 111 <-> 112 = <u>46.6%</u>
AtbHLH112 (AT1G61660)	2761	1182	394	7	X	112 <-> 27 = <u>34.2%</u> 112 <-> 111 = <u>46.6 %</u>

*Gene length includes 5' and 3'-UTRs (untranslated regions), introns, exons

**CDS length includes only exons

Figure 3.1.1: Phylogenetic relationship of select AtbHLH TFs (representative of all groups) based on full-length protein sequence. Groups are labeled (I-XII) based on structural similarities, specifically the organization of conserved domains (Heim *et al.*, 2003). bHLH27, AtbHLH111 and AtbHLH112 examined in this study are highlighted. Tree was constructed using the Maximum Likelihood method based on the JTT matrix-based model with 1000 bootstrap replicates and discrete Gamma distribution (10 categories) to model evolutionary rate differences among sites

(MEGA7 software). Tree was left unrooted (1) to define direct relationships between primary protein sequences (i.e. similarity of amino acids), (2) because no common ancestor could be assumed for the AtbHLH TFs, and (3) since the directionality of evolutionary change was not required for grouping based on structural similarities.

Figure 3.1.2: Transcriptomic expression analysis of (A) *AtbHLH27*, *AtbHLH111*, *AtbHLH112* genes and (B) genes encoding proteins with WD40 motifs (*DWA3*, *At3g50390*), based on compiled RNA-Seq data from the literature. Tissue types with the most highly expressed relative RNA transcript levels are circled in red, and root differentiation zone is circled for all gene candidates. All info and images were adapted from ProteomicsDB (Schmidt *et al.*, 2018). TPM; transcripts per million.

Figure 3.1.3: Tissue-specific root expression analysis of the *AtbHLH111*, *AtbHLH112*, and *DWA3* genes. Data is from a high-resolution spatiotemporal map created from large-scale microarray experiments and visualized on the BAR ePlant tool (Brady *et al.*, 2007). Relative expression level (REL) is relative to root tissue with the lowest detectable amount of RNA transcript. X, xylem; P, phloem

3.2: Testing of MYB/bHLH/WD40 protein-protein interactions (PPIs) using yeast 2- and 3-hybrid analyses

To determine a small PPI network of binary interactions, a targeted yeast 2-hybrid (Y2H) experiment was conducted for various MYB, bHLH, and WD40 (MBW) protein combinations. Following Y2H analysis, yeast 3-hybrid (Y3H) was used to characterize potential trimeric MBW complexes. Figure 3.2.1 illustrates two possible scenarios for trimeric MBW complexes involved in regulation of gene expression, specifically transcription. In one case (panel A), WD40-repeat motif protein acts as a protein bridge between the MYB and bHLH TFs, such that the MYB-bHLH PPI would not occur without the WD40-repeat protein. Alternatively (panel B), the MYB and bHLH TFs are able to interact with each other in the absence of a WD40-repeat protein, but addition of the WD40-repeat protein enhances the MYB-bHLH interaction or the transcriptional activity of the complex. In either situation (panels A or B), performing Y3H analysis may provide clues as to how the putative MBW complex functions, specifically if there is any enhancement effect by the candidate WD40-repeat containing proteins. It is also possible that the candidate WD40-repeat protein (i.e. At3g50390) is not involved in the MYB-bHLH interactions being investigated here, or that the WD40-repeat proteins that we have identified here as candidates are incorrect and other WD40 protein(s) are involved.

I first investigated binary PPIs between our candidate proteins (three bHLH and one WD40-repeat protein [AT3G50390]) with MYB92 or MYB93 (the other WD40-repeat protein, DWA3, was not included because its cDNA could not be obtained). To do this, the GAL4-DNA binding domain (BD) (bait) was fused to each

of the N-terminal portions of MYB92 (185 aa or 555 bp of coding region) and MYB93 (173 aa or 519 bp of coding region), which contains the R2R3 domain and some flanking amino acids (called N-MYB92 and N-MYB93 from herein). The full-length versions of these proteins contain a transactivation domain (Figure 1.6) and therefore cause activation of the reporter genes in yeast in the absence of an interacting prey protein (Sofia Khalil, personal correspondence). The N-terminal R2R3 portion of other plant MYB TFs are involved in direct interactions with other proteins, including with bHLH TFs (Feller *et al.*, 2011)(Pireyre and Burow, 2015). N-MYB53 was excluded because when its R2R3 domain was fused to the GAL4-BD, it activated the *ADE2*, *HIS3* and *MEL1* reporter genes on its own, resulting in false-positive results (Sofia Khalil, personal correspondence) (Figure S2). The full-length coding sequences of bHLH27, bHLH111, bHLH112, and the WD40-repeat protein (At3g50390) were fused to the GAL4 activation domain (AD) (preys). These bait and prey constructs were co-transformed into yeast, and cultured to grow on different minimal media to monitor reporter gene activation (*ADE2*, *HIS3*). The two additional reporter genes (*MEL1*, *AUR1-C*) were not tested during the subsequent targeted Y2H assays because the double nutritional reporter gene system (under the control of two separate promoters: *ADE2*, G2 promoter; *HIS3*, G1 promoter) was sufficient enough to confirm binary interactions between bait and prey protein constructs. The additional *MEL1* and *AUR1-C* reporter genes were designed to act as more stringent indicators of auto-activation (Sofia Khalil, personal correspondence).

As expected, the empty pGBKT7-BD bait vector (negative control, GAL4-DNA binding domain alone) in combination with bHLH prey proteins grew on control

media specific to the auxotrophic markers on the bait and prey plasmids (DDO, - Trp/-Leu), but did not grow on media lacking all four nutritional reporters (QDO, - Trp/-Leu/-Ade/-His) and therefore did not activate the *ADE2* and *HIS3* reporter genes (Figure 3.2.1 A). Likewise, no growth on the QDO media occurred for N-MYB92 or N-MYB93 bait proteins when combined with bHLH27 or bHLH112 prey proteins (Figure 3.2.1 A). Lack of growth on QDO media indicates no binary interaction of these proteins in yeast. Additionally, the WD40-repeat protein (At3g50390) did not interact in yeast with N-MYB92 or N-MYB93 or the bHLH27 or bHLH111 bait proteins (Figure 3.2.1 D). The bHLH112 bait construct could not be used for Y2H testing due to auto-activation of Y2H reporter genes (*ADE2*, *HIS3*, *MEL1*, *AUR1-C*) in the absence of a protein interaction, similar to N-MYB53 bait (Figure S3).

By contrast, N-MYB92 and the N-MYB93 bait proteins in the presence of bHLH111 prey protein produced clear growth on QDO media (Figure 3.2.1 A). This indicates that the N-MYB92 or N-MYB93 proteins are each able to physically interact with bHLH111 in yeast to activate nutritional reporter genes required to grow on QDO media. To provide further support for this interaction as biologically relevant, a follow-up Y2H experiment was carried out, where the bHLH111 protein was split into two parts: the minimal predicted protein-protein interaction region (HLH) of the protein and the minimal basic DNA-binding region (b) that is not expected to be involved in a PPI with MYB proteins (Figure 3.2.1 B). The coding regions for these partial bHLH111 protein sequences were separately cloned into the prey vector, and the Y2H assay was carried out as before. As predicted, the HLH

region of bHLH111 (in the basic deletion protein) interacted with the N-MYB92 and N-MYB93 proteins, but the basic region of bHLH111 (in the HLH deletion protein) did not interact on its own (Figure 3.2.1 C).

For the analysis of potential trimeric MBW complexes, a special plasmid called pBridge was used for Y3H assays, which works on the same principle as Y2H, but with a methionine-responsive promoter controlling the expression of a third 'bridge' protein (in this case, a candidate WD40-repeat protein). The bait protein fused to the GAL4 DNA binding and the unfused 'bridge' protein are expressed from the same pBridge plasmid (Figure 3.2.3 A). The pBridge plasmid contains the *TRP1* nutritional marker for growth of yeast on minimal media lacking tryptophan. The GAL4 DNA-BD bait protein is expressed from the constitutive *ADH1* promoter and the unfused 'bridge' protein is conditionally expressed from the *MET25* promoter in response to methionine levels in the medium (repressed in the presence of 1 mM methionine and expressed in the absence of methionine). The pBridge plasmid, expressing N-MYB bait with putative WD40 bridge, and the pGADT7 prey plasmid, expressing bHLH prey, were co-transformed into host yeast cells, and then grown on minimal PDO media (-Trp/-Leu/-Met/-Ade/-His) to observe potential PPIs (Figure 3.2.3 B).

In the absence of the amino acid methionine, the WD40-repeat protein (At3g50390) was expressed to observe the effects of this third protein on the interaction of existing bait (N-MYB92 or N-MYB93) and prey (bHLH27, bHLH111 or bHLH112) fusion proteins. As expected, the empty pBridge bait vector (negative control) in combination with bHLH prey proteins always grew on control media

selecting the plasmid themselves, TDO (-Trp/-Leu/-Met), but never grew on media lacking all five nutritional reporters, PDO (-Trp/-Leu/-Met/-Ade/-His), with *ADE2* and *HIS3* being the reporter genes. Lack of growth on PDO media indicates no trimeric interaction of these proteins within yeast (Figure 3.2.3 B). In the case of all combinations, no growth occurred on PDO media, meaning that this WD40-repeat protein At3g50390 likely plays no direct role in the physical interaction of N-MYB92 or N-MYB93 with bHLH27, bHLH111 or bHLH112. Unexpectedly, the previously observed binary interaction of N-MYB92 or N-MYB93 with bHLH111 did not occur in the context of this Y3H experiment (see Discussion).

Figure 3.2.1: Principle of yeast 3-hybrid (Y3H) assay. Bait (MYB) and prey (bHLH) protein domains are fused to the yeast GAL4 DNA binding domain (BD) and the GAL4 activation domain (AD), respectively. (A) A third necessary protein (WD40-repeat protein- mediated bridge) to form a trimeric protein complex (MBW) to initiate gene expression. (B) Presence of the third protein (WD40-repeat protein) is not required for a protein-protein interaction (PPI) between bait (MYB) and prey (bHLH), but it may enhance the stability of the binary PPI or transcriptional activity of the MBW complex. It is also possible that a WD40 is not involved in the interaction between the specific MYB and bHLH proteins being studied, or that the

WD40-repeat protein was not correctly identified. UAS; upstream activation sequence.

- B**
- C-bHLH111 (N-basic deletion)
 - C-HLH region is expected to interact with MYB bait proteins, even in the absence of basic region
 - N-bHLH111 (C-HLH deletion)
 - N-basic region is not required for PPI

Figure 3.2.2: Yeast 2-hybrid (Y2H) experiment to test for MYB-bHLH and MYB-WD40 interactions. (A) N-MYB92/N-MYB93 baits with bHLH27/bHLH111/bHLH112 preys; (B) summary of rationale for bHLH111 deletion experiment; (C) N-MYB92/N-MYB93 baits with bHLH111 deletions (N or C-terminal deletions); (D) N-MYB92/N-MYB93 and bHLH27/111/112 preys with WD40 (At3g50390) prey. Yeast cell density legend represents approximate amount of cells per drop inoculation. Samples were tested in duplicate and the same results were obtained for both trials, but only one experimental set is shown here. DDO, double dropout media; QDO, quadruple dropout media; W, tryptophan (*TRP1*); L, leucine (*LEU2*); A, adenine (*ADE2*); H, histidine (*HIS3*).

Figure 3.2.3: Yeast 3-hybrid (Y3H) experiment to test for MYB/bHLH/WD40 trimeric complexes. (A) Schematics of proteins involved in this Y3H experiment; the WD40-repeat protein (At3g50390) is expressed only in the absence of methionine (-Met). (B) N-MYB92/N-MYB93 baits with bHLH/111/112 preys, in the presence of unfused WD40-repeat protein (At3g50390). Refer to Figure 3.2.1D for yeast density legend. Samples were tested in duplicate and the same results were obtained for both trials, but only one experimental set is shown here. TDO, triple dropout media; PDO, pentuple dropout media; W, tryptophan (*TRP1*); L, leucine (*LEU2*); M, methionine (*MET25*); A, adenine (*ADE2*); H, histidine (*HIS3*).

3.3: Expression patterns of *AtbHLH111* gene deduced from promoter : reporter gene assay

Since bHLH111 was found to interact with N-MYB92 and N-MYB93 in the Y2H experiment (Chapter 3.2), I further investigated the expression pattern of the *bHLH111* gene using a promoter : reporter gene (GUS) assay to determine whether its expression correlates with suberin deposition in the root endodermis. Deposition of suberin lamellae in the root endodermis begins with an intermittent “patchy” pattern in state I of root differentiation (within the differentiation zone), just above the younger undifferentiated region closest to the growing root tip. Here, only occasional groups of endodermal cells will become modified with suberin as a secondary cell wall feature; the patchy suberization gradually becomes continuous in differentiated state II, moving up toward the mature root (Andersen *et al.*, 2015).

This pattern is reflected by the expression of several suberin-associated genes, such as *FAR1*, *FAR4*, *FAR5* (Domergue *et al.*, 2010), *GPAT5* (Beisson *et al.*, 2007), *CYP86B1/RALPH* (Compagnon *et al.*, 2009), and most recently *MYB53*, *MYB92* and *MYB93* (Murmu *et al.*, manuscript in preparation)(Klein, 2019).

The 2660 bp region immediately upstream and including the start codon of the *bHLH111* gene was fused in frame with the coding region of the GUS reporter gene (i.e. translational fusion). This *bHLH111* promoter fragment is of intermediate length between two *AtbHLH112*:GUS constructs reported in the literature: 3200 bp (Wang *et al.*, 2014) and 1320 bp (Liu *et al.*, 2015). The T-DNA plasmid construct containing the *bHLH111* promoter : GUS fusion was transformed into *Agrobacterium tumefaciens*, and then *A. thaliana* wild-type plants (Col-0) were transformed by floral dip. T₂ seeds, harvested from mature T₁ plants, were plated on minimal media to grow transgenic young seedlings used for GUS staining. β -glucuronidase (GUS) is a hydrolytic enzyme that catalyzes the cleavage of β -D-glucuronide substrate (X-gluc) to a β -D-glucuronic acid product, which forms a stable blue precipitate upon dimerization (Appendix, Figure A2) (Jefferson *et al.*, 1987). Light microscopy is used to visualize the presence of blue product, which represents the tissue-specific promoter activity of the gene (i.e. *bHLH111*) *in planta*. Higher plants lack intrinsic GUS activity and therefore no background staining occurs (Jefferson *et al.*, 1987).

Histochemical staining for GUS activity was performed with 15 independent lines in the T₂ generation, and about half (8/15) produced similar expression patterns in roots. As predicted, the mature root differentiation zone (differentiated state II) of primary roots in Promoter_{bHLH111}:GUS seedlings stained continuously

blue indicative of GUS reporter activity, demonstrating that the *bHLH111* promoter is active here (Figures 3.3.1-3.3.4). For comparison, these images are shown alongside a positive control Promoter_{FAR4}:GUS seedling (Figure 3.3.2 A,D,G), since the suberin biosynthetic enzyme FAR4 is known to be expressed in root endodermis, and is used as a marker for suberin deposition (Domergue *et al.*, 2010). Patchy expression in developing young roots (differentiated state I) is apparent in representative Promoter_{bHLH111}:GUS lines (Figure 3.3.3 E,F), with similar expression patterns to Promoter_{FAR4}:GUS seedlings (Figure 3.3.3 D) and this corresponds to patchy suberin deposition typically observed in this root zone (Andersen *et al.*, 2015)(Barberon *et al.*, 2016). The staining appears to surround the vascular bundle, which is indicative that the *bHLH111* gene is expressed in endodermal cells, and possibly internal cell layers such as the periderm and vasculature (Figure 3.3.3 A-C). A transverse cross section is required to determine the exact tissue localization (see Future Directions). Approaching the root tip, very little to no GUS activity is observed, with a complete absence of blue colour within the root tip, consistent with the *FAR4* gene expression pattern (Figure 3.3.3 G,H,I). These results indicate that the *bHLH111* gene is actively expressed in the root differentiation zone, likely in the root endodermis under normal development in young *A. thaliana* seedlings. Additionally, a few Promoter_{bHLH111}:GUS lines (#1,4,8,10) demonstrated GUS activity in the vascular tissue of leaves (Figures 3.3.1, 3.3.2), but this could be the result of overstaining seedlings rather than true promoter activity. However, it is entirely possible that *bHLH111* is actively expressed in hypocotyl leaf vascular tissue, as indicated by transcriptomics analysis (Figure 3.1.2 A).

Figure 3.3.1: Overview bright-field light microscopy images for 12-day-old GUS-stained T₂ seedlings of Promoter_{FAR4}:GUS line (A), and two representative Promoter_{bHLH111}:GUS lines (B,C). GUS staining was performed for 22 hours, destained in 70% ethanol for one week, and then imaged using a stereomicroscope. (magnification = 3X, scale bar = 2mm)

Figure 3.3.2: Bright-field light microscopy images for 12-day-old GUS-stained T₂ seedlings of additional Promoter_{bHLH111}:GUS lines showing promoter activity in roots. GUS staining was performed for 22 hours, destained in 70% ethanol for one week, and then imaged using a stereomicroscope. (magnification = 3X; scale bar = 2mm)

Figure 3.3.3: Compound light microscopy image for 12-day-old GUS-stained T₂ seedlings of Promoter_{FAR4}:GUS line (A,D,G) and two representative Promoter_{bHLH111}:GUS lines (B,C,E,F,H,I), showing promoter activity in roots. GUS staining was performed for 22 hours, destained in 70% ethanol for one week, and then imaged using a compound microscope. (magnifications = 10X, 20X; scale bars = 100µm, 50µm). Ep, epidermis; C, cortex; En, endodermis; P/V, pericycle and vascular cylinder.

Figure 3.3.4: Compound light microscopy image for 12-day-old GUS-stained T₂ seedlings of additional Promoter_{bHLH111}:GUS lines showing promoter activity in roots. GUS staining was performed for 22 hours, destained in 70% ethanol for one week, and then imaged using a compound microscope. (magnification = 10X; scale bar = 100μm)

3.4: Analysis of root suberin in mutants affected in *Arabidopsis* genes encoding BHLH and WD40-repeat proteins

To investigate the effects of homozygous loss-of-function mutations in the genes *bHLH27*, *bHLH111*, *bHLH112*, *DWA3* (encodes a WD40-repeat protein) and *AT3G50390* (encodes a WD40-repeat protein), T-DNA insertion lines for each gene were obtained from the Arabidopsis Biological Resource Centre (arabidopsis.org) (Appendix, Table A1). If any of these genes are involved in positively regulating suberin deposition in roots, then knock-out mutations would be expected to have altered suberin levels or altered suberin monomer composition. Before using any of these T-DNA insertion lines for experimentation, homozygous plants (i.e. lines with the T-DNA insertion on both homologous chromosomes) needed to be identified using PCR-based genotyping as described using an example in Figure 3.4.2. Out of the eight T-DNA insertion lines tested, only five were identified as homozygous for the T-DNA insertion (*bhlh27-1*, SAIL_104_C12; *bhlh111-2*, SALK_001970; *bhlh112-1*, SALK_148540; *dwa3-1*, SALK_046984; *at3g50390-2*, SAIL_255_C01). Nonetheless, one homozygous T-DNA mutant was identified for each of the five genes of interest. It is generally expected that a disruption of a gene with a T-DNA insertion will result in a recessive, knockout-out mutation, especially those with T-DNA inserts in an exon (which was the case for all the T-DNA lines characterized here; Figure 3.4.1). However, it is still possible that a T-DNA insertion in an exon does not result in a knock-out mutation, especially if the T-DNA is located towards the end of the coding region of the gene (O'Malley *et al.*, 2015).

Whole roots from 14-day old seedlings (primary growth stage) grown on tissue culture medium were harvested and soluble lipids removed by repeated solvent extractions with chloroform/methanol. The polyaliphatic suberin polymer was depolymerized by base-catalyzed transmethylation. Suberin monomers were identified using GC-MS and quantified using GC-FID through comparison to internal standards. The major chemical classes typically found in root suberin were identified (i.e. fatty acids, ω -hydroxy fatty acids, primary fatty alcohols, dicarboxylic fatty acids, and *trans*-ferulate). It was assumed that all detected C16-C18 fatty acids were extracted from the suberin polymer, and were not a result of contamination from cellular membrane lipids during the chloroform/methanol delipidation process of roots. Also, acetylated C20:0 primary fatty alcohol and C18:2 dicarboxylic fatty acid derivatives co-eluted in the same peak for GC-FID, but were still used to calculate total suberin content, regardless that we don't know the individual amounts of these two because we don't know the relative contributions of each of these two monomers to the peak area. This is less relevant for total suberin content because all peaks were combined, and it would be sufficiently accurate for the total value considering how many compounds are contributing to the total; C20:0 fatty alcohol and C18:2 DCA are not major suberin monomers.

The *bhlh111-2* (SALK_001970) and *bhlh112-1* (SALK_148540) T-DNA mutants had reduced total content of root suberin monomers compared to wild-type (10.3% and 6.4% lower, respectively) (Figure 3.4.3). The *bhlh27-1* (SAIL_104_C12) mutant displayed suberin levels almost equal to wild-type (0.8% decrease), whereas *dwa3-1* (SALK_046984) and *at3g50390-2* (SAIL_255_C01) had

slightly, but not significantly, increased total suberin content compared to wild-type (8.0% and 5.4% higher, respectively) (Figure 3.4.3).

When divided into chemical classes, the relative abundance of each major group can be visualized (Figures 3.4.4-3.4.6). The most abundant lipid category is the ω -hydroxy (1, ω -OH) fatty acids, followed by fatty acids, dicarboxylic fatty acids (DCAs), primary fatty alcohols, and the least abundant *trans*-ferulate. The only group with a statistically significant decrease were the primary fatty alcohols for the *at3g50390-2* mutant ($p < 0.05$) (Figures 3.4.6 C, 3.4.11, 3.4.12). Across the individual fatty acids monomers, *bhlh111-1* displayed the most significant overall decrease, specifically with long-chain (C16:0, C18:2, C18:1) and very long-chain (C24:0) fatty acids, with reductions of 35.7%, 62.1%, 54.5%, and 24.2% respectively (Figure 3.4.7). The *bhlh112-1* mutant had a significantly decreased level of C20:0 fatty acid compared to wild-type (47.6%), and a significantly increased C18:1 fatty acid content (35.3%). The *wd40* T-DNA lines (*dwa3-1*, *at3g50390-2*) contained consistently greater fatty acid content compared to wild-type (7.4%-40% increase), with the exception of C18:0 monomers. Overall, *bhlh27-1* experienced the greatest increase in fatty acid content (10.7%) (Figure 3.4.7).

Only the *bhlh111-2* mutant line showed evidence for a significant reduction ($p < 0.05$) in long-chain 1, ω -OH fatty acid monomers (C16:0, C18:1, C20:0) compared to wild-type, with decreases of 32.3%, 16.5%, and 36.4%, respectively (Figure 3.4.8). An interesting differential observation was made for the primary fatty alcohols, where *bhlh27-1* and *bhlh112-1* exhibited 27.8% and 22.2% lower values for C18:0 monomers, but had 47.8% and 59.0% higher values for C22:0 monomers

(Figure 3.4.9). Additionally, *bhlh111-2* displayed a significantly increased level of C18:0 monomer (23.5%), while *at3g50390-2* fatty alcohol levels were reduced by 14.3% for C18:0 fatty alcohols and 9.3% for C22:0 fatty alcohol with a very small standard deviation for both, making this WD40 T-DNA line significantly reduced in total primary fatty alcohol ($p < 0.05$) (Figure 3.4.9). Only the long-chain DCAs (C16:0, C18:1, C18:0) were significantly affected for a variety of the tested *loss-of-function* mutants (Figure 3.4.10). Of the three *bhlh* lines, only *bhlh112-1* was significantly reduced in content for all of the long-chain DCAs, with 23.2%, 26.3%, and 18.3% decreases, respectively. Although the other *bhlh* mutants had varying decreases in long-chain DCA levels, the only significant changes were seen for *bhlh27-1* C16:0 and C18:0 monomers (16.3% and 29.8% lower, respectively), and *bhlh111-1* C18:1 monomer levels (18.3% lower). In contrast, the *wd40* lines demonstrated increased amounts of long-chain DCA content, ranging from 7.1% to 15.6% higher compared to wild-type (Figure 3.4.10).

The *trans*-ferulate data were included in a separate graph (Figure 3.4.6), because these values are approximately two orders of magnitude lower than other chemical classes, and are therefore difficult to directly compare differences side-by-side with the other groups. Although not statistically significant, the *bhlh111-2* mutant line had the greatest difference of *trans*-ferulate content compared to wild-type (20.5% lower). Differences between chemical groups were variable across all genotypes (Figures 3.4.6-3.4.10), but cumulatively did not significantly affect total suberin monomer content of the major chemical classes, with the exception of *at3g50390-2* fatty alcohols (Figures 3.4.4, 3.4.5). The results indicate that these

tested *loss-of-function* mutants have similar suberin monomer composition to wild-type *A. thaliana*.

Figure 3.4.1: Schematic structures for genes encoding bHLH27, bHLH111, bHLH112 TFs and two WD40-repeat proteins showing locations of T-DNA insertions (not to scale). Exons (numbered black boxes) and introns (black lines) are indicated between the start and stop codons, surrounded by 5'- and 3'- untranslated regions (grey boxes). T-DNA insertions are indicated with red triangles for each mutant allele, with the upstream T-DNA insertion site indicated in base pairs (bp) relative to the A (+1 bp) of the start codon.

Figure 3.4.2: Example of PCR-based genotyping method used for identifying homozygous T-DNA-insertion mutant lines. Shown is a representative agarose gel, with the PCR products (presence or absence) using: (A) the gene-specific ‘LP’ and ‘RP’ primers that flank the T-DNA insertion site (the top part of the gel) and (B) the PCR products (presence or absence) using the T-DNA right border ‘BP’ primer and the gene-specific RP primer (the bottom part of the same gel). The lane numbers for the top and bottom parts of the gel (A and B) correspond to each other, such that it is the same individual plant being genotyped in a segregating population. The individual plants could be wild-type (no T-DNA) on both homologous chromosomes

at the loci being investigated, hemizygous such that the T-DNA insert is present on just one of the homologous chromosomes, or homozygous for the T-DNA insertion. The presence of an amplicon for only the 'LP + RP' PCR indicates homozygosity for the WT allele (i.e. no T-DNA in gene on either homologous chromosome). The presence of an amplicon for only the 'BP + RP' PCR indicates homozygosity for the T-DNA insertion (T-DNA insertion in gene on both homologous chromosomes). The presence of amplicons for both the 'LP + RP' and 'BP + RP' PCRs indicates hemizygosity (T-DNA on one chromosome and wild-type on the other chromosome). Lane 1, genomic DNA (gDNA) template from WT (Col-0) with no T-DNAs present (negative control); Lane 2, hemizygous T-DNA mutant (*bhlh111-1*) gDNA template (positive control); Lanes 3+12+21, 1Kb DNA ladder (DM010, FroggaBio); Lanes 4-11 and 13-20, gDNA templates from individual plants (*bhlh111-2*) of previously unknown genotypes. Agarose gel (0.7-0.8%) was run at 50V for 5 minutes, then 95V for 20-22 minutes.

Figure 3.4.3: Total suberin monomer content in roots from 14-day-old seedlings of homozygous T-DNA mutant lines compared to wild-type (Col-0). Root tissue from 60 seedlings from four separate agar plates was pooled to make an individual replicate in order to get enough material to measure suberin content (~20mg each of delipidated dried root tissue, n=4). The amounts are µg total suberin monomer content per mg of delipidated dried root tissue (DW = dry weight). Error bars represent standard deviation (SD). Student's *t*-test was performed.

Figure 3.4.4: Total suberin monomer content in roots from 14-day-old seedlings of homozygous T-DNA mutant lines compared to wild-type (Col-0), grouped by each genotype (A) and grouped into four major chemical classes, excluding *trans*-ferulate (B). Root tissue from 60 seedlings from four separate agar plates was pooled to make an individual replicate in order to get enough material to measure suberin content (~20mg each of delipidated dried root tissue, n=4). The amounts are μg total suberin monomer content per mg of delipidated dried root tissue (DW = dry weight). Error bars represent standard deviation (SD). Student's *t*-test was performed (* $p < 0.05$).

Figure 3.4.5: Total fatty acid (A), 1,ω-OH fatty acid (B), primary fatty alcohol (C), and DCA (D) content in suberin in roots from 14-day-old seedlings of homozygous T-DNA mutant lines compared to wild-type (Col-0). Root tissue from 60 seedlings from four separate agar plates was pooled to make an individual replicate in order to get enough material to measure suberin content (~20mg each of delipidated dried root tissue, $n=4$). The amounts are μg total suberin monomer content per mg of delipidated dried root tissue (DW = dry weight). Error bars represent standard deviation (SD). Student's *t*-test was performed (* $p < 0.05$).

Figure 3.4.6: *Trans*-ferulate monomer content in roots from 14-day-old seedlings of homozygous T-DNA mutant lines compared to wild-type (Col-0). Root tissue from 60 seedlings from four separate agar plates was pooled to make an individual replicate in order to get enough material to measure suberin content (~20mg each of delipidated dried root tissue, n=4). The amounts are µg total suberin monomer content per mg of delipidated dried root tissue (DW = dry weight). Error bars represent standard deviation (SD). Student's *t*-test was performed.

Figure 3.4.7: Fatty acid content (by chain length) in suberin in roots from 14-day-old seedlings of homozygous T-DNA mutant lines compared to wild-type (Col-0).

Root tissue from 60 seedlings from four separate agar plates was pooled to make an individual replicate in order to get enough material to measure suberin content (~20mg each of delipidated dried root tissue, n=4). The amounts are µg total suberin monomer content per mg of delipidated dried root tissue (DW = dry weight). Error bars represent standard deviation (SD). Student's *t*-test was performed (*p<0.05, **p<0.01).

Figure 3.4.8: 1,ω-OH fatty acid content (by chain length) in suberin in roots from 14-day-old seedlings of homozygous T-DNA mutant lines compared to wild-type (Col-0). Root tissue from 60 seedlings from four separate agar plates was pooled to make an individual replicate in order to get enough material to measure suberin content (~20mg each of delipidated dried root tissue, n=4). The amounts are μg total suberin monomer content per mg of delipidated dried root tissue (DW = dry weight). Error bars represent standard deviation (SD). Student's *t*-test was performed (*p<0.05).

Figure 3.4.9: Primary fatty alcohol content (by chain length) in suberin in roots from 14-day-old seedlings of homozygous T-DNA mutant lines compared to wild-type (Col-0). Root tissue from 60 seedlings from four separate agar plates was pooled to make an individual replicate in order to get enough material to measure suberin content (~20mg each of delipidated dried root tissue, n=4). The amounts are µg total suberin monomer content per mg of delipidated dried root tissue (DW = dry weight). Error bars represent standard deviation (SD). Student's *t*-test was performed. (* $p < 0.05$, ** $p < 0.01$).

Figure 3.4.10: DCA content (by chain length) in suberin in roots from 14-day-old seedlings of homozygous T-DNA mutant lines compared to wild-type (Col-0). Root tissue from 60 seedlings from four separate agar plates was pooled to make an individual replicate in order to get enough material to measure suberin content (~20mg each of delipidated dried root tissue, n=4). The amounts are µg total suberin monomer content per mg of delipidated dried root tissue (DW = dry weight). Error bars represent standard deviation (SD). Student's *t*-test was performed. (* $p < 0.05$, ** $p < 0.01$).

Figure 3.4.11: Total suberin monomer content in roots from 14-day-old seedlings of homozygous T-DNA mutant lines compared to wild-type (Col-0), divided into the five major chemical groups. Root tissue from 60 seedlings from four separate agar plates was pooled to make an individual replicate in order to get enough material to measure suberin content (~20mg each of delipidated dried root tissue, n=4). The amounts are µg total suberin monomer content per mg of delipidated dried root tissue (DW = dry weight). Error bars represent standard deviation (SD). Student's *t*-test was performed.

3.5: Seed viability assays for test of lethality in select homozygous T-DNA mutant lines

As described above (Chapter 3.4), the absence of mutant homozygous plants for certain T-DNA mutant lines (*bhlh111-1*, *bhlh111-3*, and *at3g50390-1*) suggested that these T-DNA alleles may be lethal when homozygous. To investigate this further, physical aspects of these lines were studied for any signs of seed or seedling inviability to explain the inability to isolate homozygotes for these T-DNA insertions. Firstly, PCR-based genotyping was performed to monitor segregation ratios based on simple Mendelian genetics (Figures 3.5.1, 3.5.2). The results display 1:2:0 (WT:hemizygous:homozygous) ratios for the *bhlh111-1* and *bhlh111-3* T-DNA lines, indicative of homozygous lethality. The ratio for *bhlh111-1* was 5:11:0 (Figure 3.5.1), and 6:10:0 for *bhlh111-3* (Figure 3.5.2). The probability of randomly observing zero homozygous samples in a population of 16 individuals is very low $[(0.25^{16}) * 100 = 0.0000000233 \% = 2.33 * 10^{-8} \%,$ so the genotyping results are not likely to have occurred by chance. For unknown reasons, even following weeks of troubleshooting, PCR products of *at3g50390-1* never appeared on agarose gels. Due to the inability to genotype with this primer set, no conclusions could be made for the *at3g50390-1* (SAIL_513_C01) allele.

No obvious differences appeared in the external morphology of mature seeds derived from a hemizygous plant for either *bhlh111-1* or *bhlh111-3* (Figure 3.5.3). Also, there was no evidence of abnormal seed distribution within mature siliques and no aborted seeds were found, as they were all green and swollen (Figures 3.5.4, 3.5.5). Visual differences between individual seeds are the same as that observed for

wild-type seeds. Several siliques (~20 each genotype) and seeds (~100 each genotype) were analyzed and no differences were apparent, so only the best representative quality images were chosen to be included in Figures 3.5.3-5.

Although external observations of mature seeds and immature seeds in siliques provided no evidence of embryo abortion, it was still a possibility that seeds could develop, but could not germinate. To visually observe seed viability ratios in large populations of seeds, a colorimetric seed viability test, tetrazolium red (TZ), was performed alongside a seed germination test (Figure 3.5.6 & Table 3.5.1). Triphenyl tetrazolium chloride (TTC) is a redox indicator molecule that penetrates seed coats. The TZ assay is indicative of internal embryo viability, with red colouration representing the presence of living tissues capable of cellular respiration, since metabolically active tissues contain various dehydrogenases that reduce the TTC molecule into a red formazan precipitate (Appendix, Figure A3). In combination with seedling germination rates (Table 3.5.1), the TZ-stained seeds (Figure 3.5.6) clearly showed that *bhlh111-1* and *bhlh111-3* seeds are fully viable. Again, only a representative subset of the total seeds observed (~100 per genotype) were chosen to be included in the Figure 3.5.6 because no visual differences occurred between stained seeds within a given genotype. There must be some other explanation for the absence of homozygous *bhlh111-1* and *bhlh111-3* plants during PCR-based genotyping, perhaps effects on early seedling establishment for these two independent T-DNA insertion alleles (see Discussion).

Figure 3.5.1: PCR genotyping of seeds derived from individual *bhlh111-1* (SALK_065747) T-DNA insertion mutant to determine zygosity. Shown is a representative agarose gel, with the PCR products (presence or absence) using: (A) the gene-specific 'LP' and 'RP' primers that flank the T-DNA insertion site (the top part of the gel) and (B) the PCR products (presence or absence) using the T-DNA right border 'BP' primer and the gene-specific RP primer (the bottom part of the same gel). The lane numbers for the top and bottom parts of the gel (A and B) correspond to each other, such that it is the same individual plant being genotyped in a segregating population. The individual plants could be wild-type (no T-DNA) on both homologous chromosomes at the loci being investigated, hemizygous such that the T-DNA insert is present on just one of the homologous chromosomes, or

homozygous for the T-DNA insertion. The presence of an amplicon for only the 'LP + RP' PCR indicates homozygosity for the WT allele (i.e. no T-DNA in gene on either homologous chromosome). The presence of an amplicon for only the 'BP + RP' PCR indicates homozygosity for the T-DNA insertion (T-DNA insertion in gene on both homologous chromosomes). The presence of amplicons for both the 'LP + RP' and 'BP + RP' PCRs indicates hemizyosity (T-DNA on one chromosome and wild-type on the other chromosome). Lane 1, genomic DNA (gDNA) template from WT (Col-0) with no T-DNAs present (negative control); Lane 2, hemizygous T-DNA mutant (*bhlh111-1*) gDNA template (positive control); Lanes 3+12+21, 1Kb DNA ladder (DM010, FroggaBio); Lanes 4-11 and 13-20, gDNA templates from individual seedlings (F_3) derived from heterozygous parent plant (F_2). Agarose gel (0.7%) was run at 50V for 5 minutes, then 95V for 20 minutes.

Figure 3.5.2: PCR genotyping of seeds derived from individual *bhlh111-3* (SAIL_128_DO1) T-DNA insertion mutant to determine zygosity. Refer to Figure 3.5.1 for details.

Figure 3.5.3: Visual comparison of mature dried *A. thaliana* seeds comparing seeds of wild-type (A) with that of seeds derived from hemizygous *bhlh111-1* (B) or *bhlh111-3* (C) plants. Seeds were removed from dried siliques, arranged side-by-side, and imaged using a stereomicroscope. (magnification = 10X, scale bar = 1mm)

Figure 3.5.4: Visual comparison of fresh *A. thaliana* siliques comparing immature seeds of wild-type (A) with that of seeds derived from hemizygous *bhlh111-1* (B) or *bhlh111-3* (C) plants. Siliques of similar size and colour were selected from similar regions on F₃ plants of equal age and corresponding to stage 8-10 (Mizzotti *et al.*, 2018), and imaged using a stereomicroscope (Magnification = 7.5X, scale bar = 1 mm)

Figure 3.5.5: Visual comparison of dried *A. thaliana* siliques comparing immature seeds of wild-type (A) with that of seeds derived from hemizygous *bhlh111-1* (B) or *bhlh111-3* (C) plants. Siliques of similar size and colour were selected from similar regions on F₃ plants of equal age and corresponding to stage 12-14 (Mizzotti *et al.*, 2018), and imaged using a stereomicroscope. (Magnification = 7.5X, scale bar = 1 mm)

Table 3.5.1: Average germination rates for wild-type seeds and seeds derived from hemizygous *bhlh111-1* or *bhlh111-3* plants. For each test, about 300 seeds were plated on minimal agar media (triplicate of ~100 each) and germination was recorded after 12 days of growth. Student's *t*-test for both genotypes, and found no significant differences from wild-type germination rate (***p*<0.01).

	**Germination rate
WT (Col-0)	272/289 = 94.1%
<i>bhlh111-1</i> (SALK_065747)	286/301 = 95.0 %
<i>bhlh111-3</i> (SAIL_128_D01)	276/293 = 94.2 %

Figure 3.5.6: Examples of colorimetric tetrazolium red (TZ) seed viability test results for wild-type seeds and seeds derived from hemizygous *bhlh111-1* or *bhlh111-3* plants. Representative seeds of dead (boiled for 1 hour) (A) and alive (B) WT (Col-0) seeds are shown alongside *bhlh111-1* (C) and *bhlh111-3* mutants (D). Seeds with bright red staining were considered viable, partially stained seeds were considered abnormal or damaged, and completely unstained (pale yellow) seeds were considered non-viable. (magnification = 6X, scale bar = 1mm)

CHAPTER 4: DISCUSSION

Suberin is a complex and protective glycerolipid-based biopolyester deposited throughout the inner face of certain plant cell walls (adjacent to the plasma membrane). Suberin is involved in water uptake and retention, ion transport, and gas exchange within roots (Kolattukudy, 1984)(Bernards, 2002)(Enstone *et al.*, 2003)(Ranathunge *et al.*, 2011)(Graça, 2015)(Vishwanath *et al.*, 2015). A precise understanding of the regulatory mechanisms governing suberin production is important to understand its regulated biosynthesis and functions. Over the past decade, MYB-type transcription factors have been shown to be involved in the regulation of suberization in plants (Chapter 1.4). Previous studies have demonstrated an overlapping role for AtMYB53, AtMYB92, and AtMYB93 in the regulation of suberin monomer biosynthesis during normal development of the root endodermis in *Arabidopsis thaliana* (Murmu *et al.*, manuscript in preparation) (Hu, 2018) (Klein, 2019). The findings of my thesis provide information about AtbHLH27, AtbHLH111, AtbHLH112, and two AtWD40-repeat containing proteins, DWA3 and At3g50390, in relation to AtMYB92/AtMYB93 TF complexes. Several key results provide evidence for: (1) physical binary protein-protein interactions between N-MYB92 and N-MYB93 with bHLH111 using a yeast 2-hybrid experiment; (2) correlation of the *bHLH111* gene expression with sites of suberin deposition in young roots, similar to that of a gene encoding the FAR4 suberin biosynthetic enzyme, as revealed by promoter : reporter gene analysis; and (3) the possible involvement of *bHLH111* and *bHLH112* in the regulation of suberin production, as

demonstrated by chemical analysis of Arabidopsis root suberin using *loss-of-function* mutants.

4.1: Yeast-hybrid results demonstrate binary interactions between N-MYB92/93 and bHLH111 proteins

From the Y2H results, it is apparent that bHLH111 binds directly to N-terminal R2R3-containing regions of the MYB92 and MYB93 proteins, at least within yeast cells. The N-MYB93 + bHLH111 combination produced more yeast growth on minimal drop-out media compared to the N-MYB92 + bHLH111 combination, suggesting that the former was a result of a stronger protein-protein interaction within the yeast cells (Figure 3.2.2 A). This observation can contribute to a hypothesis that these MYBs have different relative activities and/or interact with different partners when bound to bHLH111 in a complex. Another possibility could be due to different amounts of N-MYB93 vs. N-MYB92 protein production in yeast.

An anomalous result occurred when studying the effects of the tested WD40-repeat protein (At3g50390). In the Y2H assay, N-MYB92 and N-MYB93 bait proteins each formed a binary interaction with bHLH111 prey (Figure 3.2.2 A), however, this same bait-prey interaction was not observed in the presence of unfused At3g50390 (Figure 3.2.3). One possibility could be that At3g50390 inhibits the physical interaction of N-MYB92 or N-MYB93 bait and bHLH111 prey, but this is unlikely because At3g50390 prey did not bind to either N-MYB92, N-MYB93 or bHLH111 bait proteins in Y2H (Figure 3.2.2 D). Perhaps instead, unfused At3g50390 protein is free to bind the DNA binding domain (BD) of GAL4-BD fused to the bait protein,

thereby preventing the MYB-bHLH interaction. Additionally, if At3g50390 has the ability to bind GAL4-AD, it could block transcription by disrupting the activity of the activation domain (AD). Further investigation is required to fully understand these findings.

It is known that AtMYB93 is a negative regulator of lateral root development in the root endodermis of *A. thaliana* (Gibbs and Coates, 2014)(Gibbs *et al.*, 2014), and suberization is affected by lateral root emergence (Li *et al.*, 2017). Since bHLH111 was shown to bind directly with N-MYB93 in yeast (Figure 3.2.2), these proteins may have the ability to interact in larger TF complexes that regulate suberin deposition under conditions of lateral root development. AtbHLH112 has also been shown to suppress lateral root emergence in *A. thaliana* when overexpressed (Wang *et al.*, 2014), so AtbHLH112 could also be involved with AtbHLH111 and AtMYB93, either directly or indirectly. Recently, the functional role of AtbHLH112 TF was characterized, in relation to stress response (Wang *et al.*, 2014)(Liu *et al.*, 2015). It was discovered that the transcriptional activation of bHLH112 is induced by drought, high salinity, and abscisic acid (ABA). Through the binding of GCG- or E-box DNA sequences, bHLH112 positively regulates expression of genes involved in physiological responses to enhance stress tolerance. This induced resistance to drought, salt, and hormonal stress is a result of the increased ability of bHLH112 to activate target genes, possibly through the recruitment of specific TF complexes (Liu *et al.*, 2015). Suberization is induced by these same stresses (Kolattukudy and Cottle, 1982)(Barberon *et al.*, 2016)(Krishnamurthy *et al.*, 2009)(Franke and Schreiber, 2007).

Given this information, bHLH27 and bHLH112 may be more involved with stress responses than normal developmental deposition of suberin in the root endodermis, and they only form PPIs with our MYBs upon an external stress signal, such as drought, high salinity, or even wounding in leaves. It was recently discovered that AtMYB53 is a master regulator of suberization in roots, and it can cause ectopic suberin deposition in leaves when overexpressed in this organ (Murmu *et al.*, manuscript in preparation)(Hu, 2018). If MYB53 is tested for PPIs in the future and found to interact with bHLH27 or bHLH112, it may be possible that these proteins are part of MYB53-TF complexes that regulate suberin biosynthesis genes in response to leaf wounding stress. Bioinformatics analysis demonstrates presence of *bHLH27* and *bHLH112* transcripts in hypocotyl leaves (Figure 3.1.2), and literature has shown gene expression of *bHLH112* in both hypocotyl leaves and mature leaves (Liu *et al.*, 2015). In a study of roots, AtbHLH27 and bHLH112 were found to be dehydration stress memory proteins, meaning that they are more likely to interact with other TFs when plants experience long periods of drought (Ding *et al.*, 2013). One of the main activities for this class of proteins is the regulation of pathways for re-adjusting osmotic and ionic equilibrium to maintain homeostasis in roots. Suberization falls into this category of stress response, so it is entirely possible that an external signal is required to mediate PPIs via post-translational modifications (PTMs) between bHLH27 and bHLH112 with MYB53, MYB92 and MYB93 in the positive regulation of suberin deposition.

Another explanation for the bHLH27 and bHLH112 data (this study and the literature) is based on nitrogen-associated metabolism and growth, where

metabolite feedback is used to control perturbations in nutrient transport within roots (Gaudinier *et al.*, 2018). This is related to manganese ion availability, because manganese levels in the soil can affect the extent of suberization and subsequently the transport of other ions (calcium, potassium, sodium, sulfur) into the stele from the cortex (Barberon *et al.*, 2016)(Chen *et al.*, 2019). Indeed, fluctuations in nutrient availability, in addition to ABA and salt stress, can trigger variable mechanisms of regulation, possibly for the stress-responsive bHLH112 TF (Wang *et al.*, 2014). This model of environmental stress regulation could also apply to bHLH27 and bHLH111, in addition to their speculated roles in normal developmental suberin regulation.

It has been observed that AtbHLH27 and AtbHLH25 heterodimerize in roots, but only upon a stress signal (Jin *et al.*, 2011). In this case, stress was caused by a parasitic cyst nematode (*Heterodera schachtii*) invading the host root. Ectopic expression of protein constructs in onion followed by bimolecular fluorescence complementation (BiFC) revealed *in planta* interaction of bHLH25 and bHLH27, which was supported by Y2H analysis (Jin *et al.*, 2011). The bHLH25 protein (At4g37850) could potentially be a new candidate protein involved in MYB complex for stress-related regulation of suberin production. Notably, AtMYB41 is a stress-induced master regulator of suberization in roots and leaves (Kosma *et al.*, 2014), and so it is possible that bHLH27, bHLH111 and bHLH112 are also involved in these stress-responsive TF complexes. Interestingly, it is hypothesized that the recruitment of MYB27 to MBW complexes for anthocyanin biosynthesis in several eudicots is facilitated through dimerized bHLH proteins present within the MBW complex (Albert *et al.*, 2014). This dimerization mechanism may translate to

Arabidopsis root endodermis and suberin biosynthesis, where dimerization of bHLH factors enhances or makes possible the binding of MYBs to activate transcription of genes encoding suberin biosynthetic enzymes or transporters.

4.2: Root expression patterns of the *bHLH111* gene are consistent with a role in suberin deposition

Promoter : reporter gene (GUS) experiments demonstrated that the *bHLH111* promoter is active in the root differentiation zone (Figure 3.3.1-3.3.4), and these findings generally agree with our original bioinformatics analysis (Figure 3.1.2, 3.1.3). From this evidence, the *bHLH111* gene is likely co-expressed with the *MYB53*, *MYB92*, *MYB93* genes, since their promoters also direct expression in the root endodermis (Klein, 2019). It thus appears that *bHLH111* is expressed in the root, at least in close proximity to the root endodermis and pericycle/vascular cylinder, but until transverse cross-sections are visualized with microscopy (Klein, 2019), the exact tissue layers are not known. Even if *bHLH111* were not found to be expressed in the endodermis under normal development, it is possible that there is induced expression of bHLH111 protein or *bHLH111* mRNA in the endodermis and other surrounding tissues upon a stress response. For example, ABA and salt stressors have been shown to induce expression of *AtMYB41* and *AtGPAT5* genes in both endodermal and surrounding cortical cells, sometimes even extending into the epidermis (Kosma *et al.*, 2014)(Barberon *et al.*, 2017). Nonetheless, the *bHLH111* expression patterns appears very similar to the *FAR4* gene, which encodes an

enzyme for suberin monomer biosynthesis, specifically C20:0 primary fatty alcohols, and is an indicator of suberin deposition (Domergue *et al.*, 2010).

From the study of *AtbHLH25/AtbHLH27* heterodimerization, it was shown by promoter : reporter gene fusions that the *bHLH25* and *bHLH27* genes are expressed in roots, first in root primordia and then within the differentiation zone later in development (Jin *et al.*, 2011). In another study, the *bHLH112* promoter was found to be active in all major plant organs (roots, shoots, flowers, seeds) of all ages (Liu *et al.*, 2015). Expression of *bHLH112* in the root differentiation zone, enriched in the region of endodermis and pericycle/vascular cylinder (Wang *et al.*, 2014) agrees with our expression analysis for *bHLH111* (Figure 3.3.3). If *bHLH111* and *bHLH112* are indeed co-expressed with the suberin regulatory MYB TFs, then this is further evidence for these bHLH TFs to be involved in suberin gene regulation. Also, it is possible that *AtbHLH111* and *AtbHLH112* function in an overlapping or partially redundant fashion similar to that of *AtMYB53*, *AtMYB92* and *AtMYB93*.

A disadvantage of staining using the GUS reporter gene is the possibility of over-staining seedlings, which results in artifactual staining of aerial tissues (e.g. leaf veins), so blue colouration in leaves may not be an accurate representation of promoter activity. However, staining in shoots cannot always be assumed as unreliable. Based on transcriptomic analysis, it is unlikely that the *bHLH111* gene is highly expressed in shoots, although there is a possibility that low levels of transcripts are produced in aerial tissues (Figure 3.1.2). MS-based proteomics data from ProteomicsDB suggests that there are detectable levels of bHLH111 protein only in roots (Schmidt *et al.*, 2018), so perhaps low levels of transcripts could be

synthesized in shoots, then transported to roots for translation into functional protein. This process of long-distance mRNA transport via phloem vessels is a well-known phenomenon in higher plants (Kehr and Kragler, 2018).

4.3: Chemical analyses of roots from T-DNA mutant plants suggest the possible involvement of bHLH proteins in suberin biosynthesis regulation

Analysis of suberin monomer levels and composition indicated that the *bhlh111-2* (SALK_001970) and *bhlh112-1* (SALK_148540) T-DNA mutants have suberin-related phenotypes, since they both had a decrease in total root suberin content compared to wild-type *A. thaliana* (10.3% and 6.4% decreases, respectively) (Figure 3.4.3). These values are less drastic compared to *myb53*, *myb92* or *myb93* *loss-of-function* mutants, with reductions of 20-27% in the single knock-out lines (Hu, 2018). These results, in combination with our other findings (Chapters 4.1, 4.2), further support the hypothesis that bHLH TFs have regulatory activity affecting suberin production in young *Arabidopsis* roots, likely acting with MYB TFs.

Loss-of-function mutations for the tested genes can possibly affect lipid metabolism, contributing to perturbations in the total amount of long-chain fatty acid precursors available for synthesis of suberin-specific fatty acid monomers, which would cause differences in various carbon chain lengths (Figure 3.4.7). The *bhlh111-2* line demonstrated a trend of decreased mono-functional fatty acid content of varying chain lengths, compared to wild-type. This effect on long-chain (C16:0, C18:2, C18:1) and very long-chain (C22:0, C24:0) fatty acids is possibly related to the CYP86A1/HORST and CYP86B1/RALPH enzymes, respectively, since

differential activity of these enzymes may affect substrate levels of fatty acids that are then converted into ω -hydroxy fatty acids via the action of these cytochrome P450 hydroxylases within the endoplasmic reticulum (Vishwanath *et al.*, 2015). Although overall ω -OH fatty acid content was not significantly affected in our study, the long-chain ω -OH fatty acid monomers (C16:0, C18:1) displayed a significant decrease in the *bhlh112-1* mutant (Figure 3.4.8), indicating that CYP86A1/HORST activity may be altered in this *loss-of-function* mutant. The majority (>90%) of ω -OH fatty acids and DCAs in roots are part of the suberin polymer, with very small amounts present in the non-polymeric (soluble) fraction (Vishwanath *et al.*, 2013). In a model of suberin as an extended polyaliphatic network (Figures 1.2, 1.3), both ω -hydroxy fatty acids and DCAs would contribute to the extension of a cross-linked polymer, due to the presence of two functional groups on each molecule that could be involved in ester bond formation with glycerol or each other. In contrast, mono-functional fatty acids and primary fatty alcohols each contain only a single functional group per monomer, which prevent further extension of the cross-linked suberin polymer (Vishwanath *et al.*, 2013). Therefore, fatty acids and fatty alcohols are more abundant in the soluble fraction of suberin, and thus have less of an impact on the study of polymerized suberin monomer content compared to ω -hydroxy fatty acids and DCAs, although they may be important for barrier formation as suberin-associated waxes.

The *bhlh112-1* line displayed a 6.4% reduction in total suberin monomer content, although the corresponding protein but did not show any evidence of physical interaction with the N-MYB92 or N-MYB93 proteins by Y2H analysis

(Figure 3.2.1 A). bHLH112 may thus be functioning in a different manner than bHLH111 to regulate suberin-associated genes, such as stress-dependent interactions with MYBs, as previously discussed (Chapter 4.1). The *bhlh27-1* mutant (SAIL_104_C12) displayed suberin monomer content equal to that of wild-type *A. thaliana*. This could be because gene expression levels for *bHLH27* are lower in the root differentiation zone (only ~32 TPM), compared to *bHLH111* or *bHLH112* (~42 TPM) (Figure 3.1.2). The difference is not seemingly large, but it could make a significant difference in terms of TF complex formation and gene regulation activity.

Similar to the *bhlh27-1* mutant, the two T-DNA mutants affecting genes encoding WD40-repeat proteins, *dwa3-1* (SALK_046984) and *at3g50390-2* (SAIL_255_C01) did not display any significant decrease in root suberin content. In fact, the total suberin levels of *dwa3-1* and *at3g50390-2* was slightly greater than wild-type, suggesting the possibility of repressor activity, rather than activation of regulatory protein complexes. This result makes sense for *DWA3* and *At3g50390* in terms of Kleins's transcriptomic (RNA-Seq) data; both genes were found to be up-regulated in the *myb53 myb92 myb93* TKO lines (Table 3.1.1) (Klein, 2019), meaning these genes may be less active in the process of increased root suberization via *AtMYB53* overexpression and more active when suberization is suppressed via triple mutants of *MYB53*, *MYB92* and *MYB93*. However, these small differences were not statistically significant, so the results cannot be interpreted with full confidence. The results demonstrated a 5.4% increase in total suberin monomer content for *at3g50390-2* compared to wild-type (Figure 3.4.3), despite the total primary fatty alcohol content for this mutant being significantly reduced by 15.6% (Figure 3.4.5).

Although statistically significant in these cases, the results involving primary fatty alcohols are likely of minor importance, since C18:0-C22:0 fatty alcohols represent only about 5% of the total aliphatic monomers found in the suberin polymer of *Arabidopsis* roots, compared to about 80% of fatty alcohols monomers that are not covalently linked to the bio-polymer (Vishwanath *et al.*, 2013). Analysis of the soluble suberin fraction provides information on membrane lipids and unlinked aliphatic or phenolic compounds associated with suberin (suberin associated waxes), whereas the polymeric (insoluble) fraction contains mostly crosslinked ω -hydroxy fatty acids and DCAs, esterified to each other and to phenolic and glycerol subunits.

Possibly, the expression levels of these proteins in roots is not high enough to elicit a noticeable effect on suberin biosynthesis; in other words, not enough protein is present to form interactions with MYB complexes *in planta*. The WD40-repeat proteins chosen for study could be the wrong ones involved (if any WD40-repeat proteins are involved), even if they were the prime candidates derived from transcriptome (RNA-Seq) analysis of MYB53 overexpression and triple *myb53 myb92 myb93 loss-of-function* mutants (Klein, 2019). Many other WD40-repeat proteins could be tested, and the transient nature of MBW complexes (Xu and Min, 2011) might also make it more difficult to find a *loss-of-function* mutant with a detectable suberin phenotype.

4.4: Lack of homozygous mutants for two *bHLH111* T-DNA mutant lines are not explained by seed inviability

I was unable to identify homozygous T-DNA mutant lines for the *bhlh111-1* (SALK_065747) or *bhlh111-3* (SAIL_128_D01) alleles, despite screening a population number that would be expected to yield homozygous plants in a segregating population. In other words, no plants with a T-DNA insertion on each homologous chromosome were recovered (Chapter 3.5). This non-Mendelian segregation pattern has been observed for several T-DNA lines, as the loss of some genes can result in reduced transmission or even seed lethality, causing the inability to isolate mutants homozygous for inserts (O'Malley *et al.*, 2015). There did not appear to be alterations in seed germination or embryo viability in the segregating population to explain this observation (Table 3.5.1; Figure 3.5.6). It is possible that the seeds for these *bHLH111* knockout lines do not develop past a very early stage of development. Aborted seeds are typically easy to spot within a group of developed seeds, but only if they reach a certain size before dying (Zhou *et al.*, 2013). Therefore, if embryogenesis is terminated very early for the homozygous offspring, then those seeds will never appear in the siliques, and their absence disguised by the growth of full healthy seeds. Embryonic transcriptome profiling shows that *bHLH111* is highly expressed during the earliest preglobular (8/16 cell) stage of embryogenesis (Hofmann *et al.*, 2019). The *bhlh111-2* T-DNA line (SALK_001970) used for phenotypic suberin analysis (Chapter 3.4) may be a knockDOWN mutant (i.e. partial *loss-of-function*) rather than a full knockOUT (i.e. full *loss-of-function*) because this T-DNA insertion allowed for the production of viable homozygous seedlings, in contrast to the *bhlh111-1* and *bhlh111-3* alleles. Another explanation for the lack of homozygous *bhlh111-1* and *bhlh111-3* alleles could be that these lines

have additional T-DNA insertions in essential genes outside of the *bHLH111* gene. There is an average of 1.13 random insertions per line in transgenic *Arabidopsis* (Koncz *et al.*, 1992)(O'Malley *et al.*, 2015). Closely linked T-DNAs within the mutant genome can co-segregate from parent to offspring. Also, since T-DNA transformation is a complex event and DNA rearrangements from insertions can disrupt proper segregation (O'Malley *et al.*, 2015). Further investigation of these uncertain T-DNA mutant lines is required to gain a better understanding of the problem.

4.5: Predicted model of bHLH111 regulatory activity

Given the above evidence supporting the involvement of *AtbHLH111* in regulation of suberin deposition in the *Arabidopsis* root endodermis, I hypothesize that AtbHLH111 TF functions in a feedback regulation manner (Figure 1.10), where the downstream *bHLH111* gene is regulated by MYB92 and/or MYB93, and bHLH111 then forms PPIs with these same MYBs to regulate itself and other genes, such as suberin-associated genes. Indeed, other feedback loops involving MYB and bHLH TFs have been found in plants, such as one for the proanthocyanidin biosynthetic pathway in poplar, where bHLH131 interacts with MYB115 or MYB134 to regulate promoters of these same *MYB* genes in a positive manner for signal amplification (James *et al.*, 2017). A related example in *A. thaliana* involves the AtCFL1 protein with two bHLH TFs, CFL-associated protein 1 (CFLAP1) and CFLAP2 (Li *et al.*, 2016). The master regulator AtCFL1 negatively regulates cuticle development by interacting with another TF, HDG1. Both CFLAP1/2 are involved in

the AtCFL1-mediated regulation pathway, and compete for the same binding site as HDG1. The bHLH TFs, CFLAP1/2, are thus positive regulators of cuticle development when they replace the negative regulator, HDG1. It is not necessarily a feedback model of gene regulation, but it exemplifies the idea of positive/negative regulation of master TFs for a given process, whether it is a developmental or stress-response pathway. The model proposed here for bHLH111 is a combination of positive feedback regulation and competitive repressor/activator gene regulation. Further studies of our AtMYB protein complexes will elucidate the molecular mechanisms controlling the regulation of suberin-associated genes.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

In summary, the findings of this study indicate that the bHLH111 protein (AT1G31050.1) is involved in the regulation of suberin-associated genes during normal development of *A. thaliana* root endodermis, via direct interactions each with the MYB92 and MYB93 TFs. This conclusion comes from several lines of evidence: 1) Y2H experiments demonstrate binary PPIs between bHLH111 prey and MYB92 or MYB93 bait proteins, within yeast cells; 2) promoter : GUS reporter assay indicates that *bHLH111* promoter is localized around the root endodermis where suberin is deposited (just like *MYB53*, *MYB92*, and *MYB93* as well as biosynthetic genes); 3) chemical analysis reveals that total suberin monomer content in young roots is reduced by 10.3% in a *loss-of-function* T-DNA line, *bHLH111-2* (SALK_001970), compared to wild-type (Col-0).

From this information, it is predicted that bHLH111 protein functions in a feedback regulation manner (Figure 1.10), where the downstream *bHLH111* gene is regulated by MYB92 and MYB93 TFs, which then forms PPIs with these same MYBs to regulate itself and other genes, such as suberin-biosynthetic or transporter genes (Figure 1.10). Similar to most TFs, bHLH111 is not necessarily limited to one biological process, and future efforts should be taken to help elucidate the various functions of this regulator. However, in the context of this study, it is seemingly an important partner of many MYB protein complexes that regulate suberin-associated genes during normal root development of *A. thaliana*.

Despite these discoveries, many follow-up experiments need to be performed to support the findings of this study, and to learn new aspects of AtbHLH111's involvement in suberin gene regulation and other processes. A few ideas for future studies are discussed below.

Not all possibilities were investigated for bait/prey combinations in the Y2H experiments, for example a WD40-repeat (At3g50390) bait (instead of prey) could be generated and tested with bHLH27, bHLH111, and bHLH112 prey proteins to test for activation of the reporter genes in this context. Also, the combination of N-MYB92 or N-MYB93 as prey with bHLH27, bHLH111, and At3g50390 as bait proteins (reverse orientation of experiment performed here) may yield positive results, and is an important follow-up for the MYB92/MYB93 bait + bHLH111 prey positive result. It is predicted that bHLH111 bait + MYB92/MYB93 prey proteins will interact in yeast, consistent with the results observed here (Figure 3.2.2 A).

Finally, full-length MYB coding sequences could be cloned into the prey constructs, tested for auto-activation and then tested with the various bHLH and WD40-repeat bait proteins.

Another approach to PPI analysis is the use of BiFC or split-luciferase assays. These advanced fluorescent imaging techniques are used to detect PPIs *in planta*. They allow for non-invasive, real-time visualization of PPIs and their localization in living cells. The technology is based upon reformation of fusion proteins expressed in the cell, where split inactive fluorophores recombine to form an actively fluorescent protein interaction when they come into close proximity. Fluorescence is only achieved when binary proteins physically interact, which can be viewed by microscopy at the appropriate wavelength(s) for detection (Hu *et al.*, 2002; Kerppola, 2006; Ohad *et al.*, 2007; Schütze *et al.*, 2009). Therefore, bHLH111 and MYB53, MYB92, and MYB93 fusion proteins can all be expressed, and tested for interactions in living *A. thaliana* root cells or protoplasts. Unlike Y2H, the BiFC and split-luciferase fusion constructs could make use of full-length MYB sequences, rather than truncations (Chapters 1.6, 3.2). *In planta* methods for PPI detection have obvious advantages over yeast-hybrid because limitations are innately present for plant proteins expressed in yeast cells, such as variable post-translational modifications (PTMs). Since the MYB activation domain can probably only be regulated by plant signalling pathways, which do not occur in yeast cells, and these PTMs could change the interaction abilities of full-length MYB proteins. Additionally, these fluorescent imaging techniques work on the principle of split fluorophore recombination, so limitations related to reporter genes are not an issue, unlike

yeast-hybrid where the entire system is reliant on transcription of reporter genes. If transcription is affected by the introduction of bait/prey fusion proteins (or unfused protein in Y3H), it can result in false negative/positive results. Despite the several advantages of BiFC and split-luciferase methods, they are costly and can potentially yield high amounts of false positive and false negative artifacts during analysis, respectively (Xing *et al.*, 2016).

A complimentary experiment would be the use of co-immunoprecipitation (co-IP) to investigate the possibility of a larger protein complex involving bHLH111/MYB92 or bHLH111/MYB93. Co-IP utilizes an antibody or protein tag to capture the protein of interest, along with any bound proteins. This technique is useful for both binary interactions and native protein complexes, since a cell lysate is used instead of a library. All natural interactions have the potential to be captured by this assay. The cell lysate is incubated with coupled antibody or tag, then purified, eluted, and separated. Only the protein of interest and any physically interacting proteins should precipitate together and remain in the final solution. Binding partners can be analyzed by gel electrophoresis and mass spectrometry for subsequent identification (Lee, 2007; Van Der Geer, 2014; Lin and Lai, 2017). If bHLH111 truly interacts with MYB92 or MYB93 *in planta*, and MYBs often function in larger protein complexes, then it is probable that other interacting partners will be extracted from the co-IP experiments. A disadvantage of the co-IP technique is the use of mass spectrometry required to identify novel protein interactors in the complex, which can be costly and exhaustive if dealing with large TF complexes. In addition, co-IP is not high-throughput, and is not suitable for detecting transient

interactions, which can limit the ability to accurately characterize protein complexes that occur *in planta* (Xing *et al.*, 2016).

As mentioned in Chapter 4.2, it is essential to verify that the *AtbHLH111* promoter is active in the root endodermis. Based on the data presented here, both bioinformatics and promoter : GUS analysis, it is likely that *bHLH111* is expressed in the root endodermis (co-expressed with *MYB53*, *MYB92*, and *MYB93*) and possibly the pericycle/vascular bundle, but transverse cross-sections must be obtained and imaged to confirm exact tissue-specific expression (Klein, 2019). It is also worth obtaining promoter : reporter gene constructs for *bHLH27* and *bHLH112* and generating transgenic *A. thaliana*, in order to gather information about their precise expression patterns and potential for overlapping function with *bHLH111*. The reporter gene activity for the *bHLH112* promoter is expected to resemble that of the *bHLH111* promoter, since other studies have utilized promoter : reporter gene analysis and RT-PCR to demonstrate *bHLH112* expression in several plant organs and tissues, including root pericycle, stele and possibly root endodermis (Wang *et al.*, 2014)(Liu *et al.*, 2015). Based on RNA-Seq and microarray data (Chapter 3.1), I predict that the bHLH27 and bHLH112 proteins are both expressed in the root differentiation zone, but bHLH112 is more involved with suberin gene regulation because of our results in Chapter 3.4, where total suberin monomer content is slightly reduced in the bHLH112 *loss-of-function* mutant, but not the bHLH27 *loss-of-function* mutant.

It would be beneficial to perform quantitative RT-PCR for *bHLH27*, *bHLH111*, *bHLH112*, *DWA3*, and *At3g50390* isolated from several *Arabidopsis* organs (roots,

young leaves, old leaves, inflorescence stem, siliques, flowers, etc.) to quantify relative transcript levels for these candidate genes during normal development. Additionally, chemical stressors such as addition of stress hormone abscisic acid (ABA) or high salt concentration, or physical stress such as drought and wounding, can be implemented to test their effects on *bHLH27*, *bHLH111*, *bHLH112*, *DWA3*, and *At3g50390* transcript levels in roots, as these stresses have been shown to alter suberin deposition (Kolattukudy and Cottle, 1982)(Barberon *et al.*, 2016)(Krishnamurthy *et al.*, 2009)(Franke and Schreiber, 2007)(Schreiber *et al.*, 2005). It is possible that our proteins of interest respond to stress, with functions beyond those of normal root development.

In support of this study, additional T-DNA mutant lines for the *bHLH111* and *bHLH112* genes should be tested for suberin monomer levels. If at least two independent lines display a reduced root suberin phenotype, then we can be more confident in our conclusion to further pursue these as important regulators relevant to suberin deposition. It would also be of interest to observe how a *bhlh111 bhlh112* double KO mutant would affect suberin monomer composition. CRISPR-Cas9 could also be an option for creating stable *loss-of-function* lines for more in-depth studies. Another worthwhile phenotypic test for suberin reduction is a fluoryl yellow staining of roots, since this dye targets the aliphatic domains of the suberin polymer, resulting in a fluorescent signal when viewed with microscopy (Andersen *et al.*, 2018)(Mahmood *et al.*, 2019)(Cohen *et al.*, 2020). The change in suberin can then be visually observed through development, if the reduction is drastic enough.

The above materials and experiments could provide more concrete evidence for the involvement of bHLH111, working with the MYB53, MYB92 and MYB93 TFs, in the regulation of suberin-associated genes during normal development of *A. thaliana* root endodermis. The above points are recommendations for future studies related to my findings.

Suberin is a specialized hydrophobic barrier with several essential functions in plant development and stress response. Suberized tissue layers mediate water uptake, ion transport, and gas exchange within roots, and the degree of root suberization influences water relations and stress tolerance. For these reasons, suberin has become an increasingly important subject for research in plant science, particularly relating to crop health and environmental stress resistance. It has been found that certain transcription factors are master regulators of suberin biosynthesis genes, and alteration of these genes significantly change suberin content in various tissues, including roots, where suberin is deposited throughout normal development and under stress conditions.

These results contribute to our understanding of the molecular mechanisms by which MYB protein complexes control suberin biosynthesis and deposition in roots. This information is important for the future generation of sustainable metabolically engineered crops that are more stress resistant, via enhancement of suberin deposition at certain tissue interfaces. Environmental stress can cause major yield loss of harvested crops, and improvement of stress tolerance can mitigate, and potentially eliminate, the problem.

REFERENCES

- Albert, N.W., Davies, K.M., Lewis, D.H., et al.** (2014) A conserved network of transcriptional activators and repressors regulates anthocyanin pigmentation in Eudicots. *Plant Cell*, **26**, 962–980.
- Andersen, T.G., Barberon, M. and Geldner, N.** (2015) Suberization—the second life of an endodermal cell. *Curr. Opin. Plant Biol.*, **28**, 9–15.
- Andersen, T.G., Naseer, S., Ursache, R., Wybouw, B., Smet, W., Rybel, B. De, Vermeer, J.E.M. and Geldner, N.** (2018) Diffusible cytokinin repression establishes symmetry and passage cells in the endodermis. *Nature*, **555**, 529–533.
- Arabidopsis Interactome Mapping Consortium** (2011) Evidence for Network Evolution in an Arabidopsis Interactome Map. *Science*, **333**, 601–607.
- Barberon, M.** (2017) The endodermis as a checkpoint for nutrients. *New Phytol.*, **213**, 1604–1610.
- Barberon, M., Vermeer, J.E.M., Bellis, D. De, et al.** (2016) Adaptation of Root Function by Nutrient-Induced Plasticity of Endodermal Differentiation. *Cell*, **164**, 447–459.
- Baudry, A., Caboche, M. and Lepiniec, L.** (2006) TT8 controls its own expression in a feedback regulation involving TTG1 and homologous MYB and bHLH factors, allowing a strong and cell-specific accumulation of flavonoids in *Arabidopsis thaliana*. *Plant J.*, **46**, 768–779.
- Beisson, F., Li, Y., Bonaventure, G., Pollard, M. and Ohlrogge, J.B.** (2007) The Acyltransferase GPAT5 Is Required for the Synthesis of Suberin in Seed Coat and Root of *Arabidopsis*. *Plant Cell*, **19**, 351–368.
- Bemer, M., Dijk, A.D.J. van, Immink, R.G.H. and Angenent, G.C.** (2017) Cross-Family Transcription Factor Interactions: An Additional Layer of Gene Regulation. *Trends*

- Plant Sci.*, **22**, 66–80.
- Bernards, M.A.** (2002) Demystifying suberin. *Can. J. Bot.*, **80**, 227–240.
- Bhargava, A., Ahad, A., Wang, S., Mansfield, S.D., Haughn, G.W., Douglas, C.J. and Ellis, B.E.** (2013) The interacting MYB75 and KNAT7 transcription factors modulate secondary cell wall deposition both in stems and seed coat in Arabidopsis. *Planta*, **237**, 1199–1211.
- Brady, S.M., Orlando, D.A., Lee, J.-Y., Wang, J.Y., Koch, J., Dinneny, J.R., Mace, D., Ohler, U. and Benfey, P.N.** (2007) A High-Resolution Root Spatiotemporal Map Reveals Dominant Expression Patterns. *Science*, **318**, 801–806.
- Brady, S.M., Zhang, L., Megraw, M., et al.** (2011) A stele-enriched gene regulatory network in the Arabidopsis root. *Mol. Syst. Biol.*, **7**, 1–9.
- Cardon, G., Höhmann, S., Klein, J., Nettesheim, K., Saedler, H. and Huijser, P.** (1999) Molecular characterisation of the Arabidopsis SBP-box genes. *Gene*, **237**, 91–104.
- Chen, A., Husted, S., Salt, D.E., Schjoerring, J.K. and Persson, D.P.** (2019) The Intensity of Manganese Deficiency Strongly Affects Root Endodermal Suberization and Ion Homeostasis. *Plant Physiol.*, **181**, 729–742.
- Clough, S.J. and Bent, A.F.** (1998) Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J.*, **16**, 735–743.
- Cohen, H., Fedyuk, V., Wang, C., Wu, S. and Aharoni, A.** (2020) SUBERMAN Regulates Developmental Suberization of the Arabidopsis Root Endodermis. *Plant J.*, 1–17.
- Compagnon, V., Diehl, P., Benveniste, I., Meyer, D., Schaller, H., Schreiber, L., Franke, R. and Pinot, F.** (2009) CYP86B1 is required for very long chain ω -hydroxyacid and α,ω -dicarboxylic acid synthesis in root and seed suberin polyester. *Plant Physiol.*, **150**, 1831–1843.
- de Silva, N.D.** (2018) *The roles of suberin biopolymer and associated waxes in protecting*

plants against abiotic and biotic stresses. Full thesis available at:

<https://curve.carleton.ca/a67d8531-de04-481b-ad43-f940014176de>

Ding, Y., Liu, N., Virilouvet, L., Riethoven, J.J., Fromm, M. and Avramova, Z. (2013) Four distinct types of dehydration stress memory genes in *Arabidopsis thaliana*. *BMC Plant Biol.*, **13**:223.

Domergue, F., Vishwanath, S.J., Joubes, J., et al. (2010) Three *Arabidopsis* Fatty Acyl-Coenzyme A Reductases, FAR1, FAR4, and FAR5, Generate Primary Fatty Alcohols Associated with Suberin Deposition. *Plant Physiol.*, **153**, 1539–1554.

Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C. and Lepiniec, L. (2010) MYB transcription factors in *Arabidopsis*. *Trends Plant Sci.*, **15**, 573–581.

Edwards, K., Johnstone, C. and Thompson, C. (1991) A simple and rapid method for preparation of *rhizopus oryzae* genomic DNA for PCR analysis. *Nucleic Acids Res.*, **19**, 1349.

Enstone, D.E., Peterson, C.A. and Ma, F. (2003) Root endodermis and exodermis: Structure, function, and responses to the environment. *J. Plant Growth Regul.*, **21**, 335–351.

Feller, A., MacHemer, K., Braun, E.L. and Grotewold, E. (2011) Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. *Plant J.*, **66**, 94–116.

Franke, R. and Schreiber, L. (2007) Suberin — a biopolyester forming apoplastic plant interfaces. *Curr. Opin. Plant Biol.*, **10**, 252–259.

Gaudinier, A., Rodriguez-Medina, J., Zhang, L., et al. (2018) Transcriptional regulation of nitrogen-associated metabolism and growth. *Nature*, **563**, 259–264.

Geer, P. Van Der (2014) Analysis of protein-protein interactions by coimmunoprecipitation. *Methods Enzymol.*, **541**, 35–47.

- Geldner, N.** (2013) The Endodermis. *Annu. Rev. Plant Biol.*, **64**, 531–558.
- Gibbs, D.J. and Coates, J.C.** (2014) AtMYB93 is an endodermis-specific transcriptional regulator of lateral root development in arabidopsis. *Plant Signal. Behav.*, **9**, e970406.
- Gibbs, D.J., Voß, U., Harding, S. a, et al.** (2014) AtMYB93 is a novel negative regulator of lateral root development in Arabidopsis. *New Phytol.*, **203**, 1194–1207.
- Gou, J.-Y., Felippes, F.F., Liu, C.-J., Weigel, D. and Wang, J.-W.** (2011) Negative Regulation of Anthocyanin Biosynthesis in *Arabidopsis* by a miR156-Targeted SPL Transcription Factor. *Plant Cell*, **23**, 1512–1522.
- Gou, M., Hou, G., Yang, H., Zhang, X., Cai, Y., Kai, G. and Liu, C.-J.** (2017) The MYB107 Transcription Factor Positively Regulates Suberin Biosynthesis. *Plant Physiol.*, **173**, 1045–1058.
- Graça, J.** (2015) Suberin: the biopolyester at the frontier of plants. *Front. Chem.*, **3**, 1–11.
- Graça, J. and Santos, S.** (2006) Linear aliphatic dimeric esters from cork suberin. *Biomacromolecules*, **7**, 2003–2010.
- Heim, M.A., Jakoby, M., Werber, M., Martin, C., Weisshaar, B. and Bailey, P.C.** (2003) The basic helix-loop-helix transcription factor family in plants: A genome-wide study of protein structure and functional diversity. *Mol. Biol. Evol.*, **20**, 735–747.
- Hernandez, J.M., Feller, A., Morohashi, K., Frame, K. and Grotewold, E.** (2007) The basic helix-loop-helix domain of maize R links transcriptional regulation and histone modifications by recruitment of an EMSY-related factor. *Proc. Natl. Acad. Sci. U. S. A.*, **104**, 17222–17227.
- Hofmann, F., Schon, M.A. and Nodine, M.D.** (2019) The embryonic transcriptome of *Arabidopsis thaliana*. *Plant Reprod.*, **32**, 77–91.
- Hu, C.D., Chinenov, Y. and Kerppola, T.K.** (2002) Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence

- complementation. *Mol. Cell*, **9**, 789–798.
- Hu, H.** (2018) *The role of transcription factor MYB53 from Arabidopsis thaliana in the regulated production of suberin*. Full thesis available at:
<https://curve.carleton.ca/9a4f95f7-f6ba-436c-af66-cb49a0a5c865>
- James, A.M., Ma, D., Mellway, R., et al.** (2017) Poplar MYB115 and MYB134 transcription factors regulate proanthocyanidin synthesis and structure. *Plant Physiol.*, **174**, 154–171.
- Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W.** (1987) GUS fusions: ,B-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.*, **6**, 3901–3907.
- Jenkin, S. and Molina, I.** (2015) Isolation and Compositional Analysis of Plant Cuticle Lipid Polyester Monomers. *J. Vis. Exp.*, e53386.
- Jin, J., Hewezi, T. and Baum, T.J.** (2011) The Arabidopsis bHLH25 and bHLH27 transcription factors contribute to susceptibility to the cyst nematode *Heterodera schachtii*. *Plant J.*, **65**, 319–328.
- Kehr, J. and Kragler, F.** (2018) Long distance RNA movement. *New Phytol.*, **218**, 29–40.
- Kerppola, T.K.** (2006) Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. *Nat. Protoc.*, **1**, 1278–1286.
- Klein, D.** (2019) *The downstream targets of MYB-type transcription factors involved in suberin biosynthesis*. Full thesis available at: <https://curve.carleton.ca/e656b9e8-31ba-44b2-8696-e0d5359703d4>
- Ko, J.H., Jeon, H.W., Kim, W.C., Kim, J.Y. and Han, K.H.** (2014) The MYB46/MYB83-mediated transcriptional regulatory programme is a gatekeeper of secondary wall biosynthesis. *Ann. Bot.*, **114**, 1099–1107.
- Kolattukudy, P.E.** (1984) Biochemistry and function of cutin and suberin. *Can. J. Bot.*, **62**,

2918–2933.

Kolattukudy, P.E. and Cottle, W. (1982) Absciscic Acid Stimulation of Suberization. *Plant Physiol.*, **70**, 775–780.

Koncz, C., Németh, K., Rédei, G.P. and Schell, J. (1992) T-DNA insertional mutagenesis in *Arabidopsis*. *Plant Mol. Biol.*, **20**, 963–976.

Kosma, D.K., Murmu, J., Razeq, F.M., Santos, P., Bourgault, R., Molina, I. and Rowland, O. (2014) AtMYB41 activates ectopic suberin synthesis and assembly in multiple plant species and cell types. *Plant J.*, **80**, 216–229.

Krishnamurthy, P., Ranathunge, K., Franke, R., Prakash, H.S., Schreiber, L. and Mathew, M.K. (2009) The role of root apoplastic transport barriers in salt tolerance of rice (*Oryza sativa* L.). *Planta*, **230**, 119–134.

Lai, B., Du, L.N., Liu, R., Hu, B., Su, W.B., Qin, Y.H., Zhao, J.T., Wang, H.C. and Hu, G.B. (2016) Two LcbHLH transcription factors interacting with LcMYB1 in regulating late structural genes of anthocyanin biosynthesis in *Nicotiana glauca* and *Litchi chinensis* during anthocyanin accumulation. *Front. Plant Sci.*, **7**, 1–15.

Lashbrooke, J., Cohen, H., Levy-Samocho, D., et al. (2016) MYB107 and MYB9 Homologs Regulate Suberin Deposition in Angiosperms. *Plant Cell*, **28**, 2097–2116.

Lee, C. (2007) Coimmunoprecipitation assay. *Methods Mol. Biol.*, **362**, 401–406.

Legay, S., Guerriero, G., Andrić, C., Guignard, C., Cocco, E., Charton, S., Boutry, M., Rowland, O. and Hausman, J.F. (2016) MdMyb93 is a regulator of suberin deposition in russeted apple fruit skins. *New Phytol.*, **212**, 977–991.

Li, B., Kamiya, T., Kalmbach, L., et al. (2017) Role of LOTR1 in Nutrient Transport through Organization of Spatial Distribution of Root Endodermal Barriers. *Curr. Biol.*, **27**, 758–765.

Li, C.-L., Meng, D., Pineros, M.A., MAO, Y., Dandekar, A.M. and Cheng, L. (2019) A Sugar

- Transporter Takes Up both Hexose and Sucrose for Sorbitol-Modulated in vitro Pollen Tube Growth in Apple. *Plant Cell*, **32**, 449–469.
- Li, C., Ng, C.K.Y. and Fan, L.M.** (2015) MYB transcription factors, active players in abiotic stress signaling. *Environ. Exp. Bot.*, **114**, 80–91.
- Li, S., Wang, X., He, S., et al.** (2016) CFLAP1 and CFLAP2 Are Two bHLH Transcription Factors Participating in Synergistic Regulation of AtCFL1-Mediated Cuticle Development in Arabidopsis. *PLoS Genet.*, **12**, 1–27.
- Li, S. and Zachgo, S.** (2013) TCP3 interacts with R2R3-MYB proteins, promotes flavonoid biosynthesis and negatively regulates the auxin response in Arabidopsis thaliana. *Plant J.*, **76**, 901–913.
- Li, X., Duan, X., Jiang, H., et al.** (2006) Genome-wide analysis of basic/helix-loop-helix transcription factor family in rice and Arabidopsis. *Plant Physiol.*, **141**, 1167–1184.
- Lin, J.S. and Lai, E.M.** (2017) Protein–protein interactions: Co-immunoprecipitation. *Methods Mol. Biol.*, **1615**, 211–219.
- Liu, C., Long, J., Zhu, K., Liu, L., Yang, W., Zhang, H., Li, L., Xu, Q. and Deng, X.** (2016) Characterization of a Citrus R2R3-MYB Transcription Factor that Regulates the Flavonol and Hydroxycinnamic Acid Biosynthesis. *Sci. Rep.*, **6**, 25352.
- Liu, Y., Ji, X., Nie, X., et al.** (2015) Arabidopsis AtbHLH112 regulates the expression of genes involved in abiotic stress tolerance by binding to their E-box and GCG-box motifs. *New Phytol.*, **207**, 692–709.
- Ma, D., Reichelt, M., Yoshida, K., Gershenzon, J. and Constabel, C.P.** (2018) Two R2R3-MYB proteins are broad repressors of flavonoid and phenylpropanoid metabolism in poplar. *Plant J.*, **96**, 949–965.
- Mahmood, K., Zeisler-Diehl, V.V., Schreiber, L., Bi, Y.M., Rothstein, S.J. and Ranathunge, K.** (2019) Overexpression of ANAC046 Promotes Suberin Biosynthesis in Roots of

Arabidopsis thaliana. *Int. J. Mol. Sci.*, **20**.

Mattinen, M.L., Filpponen, I., Järvinen, R., Li, B., Kallio, H., Lektinen, P. and

Argyropoulos, D. (2009) Structure of the polyphenolic component of suberin isolated from potato (*Solanum tuberosum* war. Nikola). *J. Agric. Food Chem.*, **57**, 9747–9753.

McFarlane, H.E., Watanabe, Y., Yang, W., Huang, Y., Ohlrogge, J. and Samuels, A.L.

(2014) Golgi- and Trans-Golgi Network-Mediated Vesicle Trafficking Is Required for Wax Secretion from Epidermal Cells. *Plant Physiol.*, **164**, 1250–1260.

Mizzotti, C., Rotasperi, L., Moretto, M., et al. (2018) Time-course transcriptome analysis of arabidopsis siliques discloses genes essential for fruit development and maturation. *Plant Physiol.*, **178**, 1249–1268.

Molina, I., Li-Beisson, Y., Beisson, F., Ohlrogge, J.B. and Pollard, M. (2009) Identification of an Arabidopsis Feruloyl-Coenzyme A Transferase Required for Suberin Synthesis. *PLANT Physiol.*, **151**, 1317–1328.

Mossoba, M.M., Tyburczy, C., Delmonte, P., Fardin-Kia, A.R., Rader, J.I., Azizian, H. and

Kramer, J.K. (2014) Application of Gas Chromatography and Infrared Spectroscopy for the Determination of the Total Trans Fatty Acid, Saturated Fatty Acid, Monounsaturated Fatty Acid, and Polyunsaturated Fatty Acid Contents in Edible Fats and Oils. In *Trans Fats Replacement Solutions*. pp. 89–121.

Naseer, S., Lee, Y., Lapierre, C., Franke, R., Nawrath, C. and Geldner, N. (2012) Casparian strip diffusion barrier in Arabidopsis is made of a lignin polymer without suberin. *Proc. Natl. Acad. Sci.*, **109**, 10101–10106.

O'Malley, R.C.O., Barragan, C.C. and Ecker, J.R. (2015) A User's Guide to the Arabidopsis T-DNA Insertional Mutant Collections. *Methods Mol. Biol.*, **1284**, 323–342.

Ohad, N., Shichrur, K. and Yalovsky, S. (2007) The Analysis of Protein-Protein Interactions in Plants by Bimolecular Fluorescence Complementation. *PLANT Physiol.*,

145, 1090–1099.

Pesch, M., Schultheiß, I., Klopffleisch, K., Uhrig, J.F., Koegl, M., Clemen, C.S., Simon, R.,

Weidtkamp-Peters, S. and Hülkamp, M. (2015) TRANSPARENT TESTA GLABRA1

and GLABRA1 Compete for Binding to GLABRA3 in Arabidopsis. *Plant Physiol.*, **168**, 584–597.

Peterson, C.A. and Cholewa, E. (1998) Structural modifications of the apoplast and their potential impact on ion uptake. *Zeitschrift für Pflanzenernährung und Bodenkd.*, **161**, 521–531.

Pireyre, M. and Burow, M. (2015) Regulation of MYB and bHLH transcription factors: A glance at the protein level. *Mol. Plant*, **8**, 378–388.

Pollard, M., Beisson, F., Li, Y. and Ohlrogge, J.B. (2008) Building lipid barriers: biosynthesis of cutin and suberin. *Trends Plant Sci.*, **13**, 236–246.

Porter, K.R. and Kallman, F. (1953) Osmium tetroxide in electron microscopy. *Exp. Cell Res.*, **4**, 127–141.

Ramsay, N.A. and Glover, B.J. (2005) MYB-bHLH-WD40 protein complex and the evolution of cellular diversity. *Trends Plant Sci.*, **10**, 63–70.

Ranathunge, K., Schreiber, L. and Franke, R. (2011) Suberin research in the genomics era — New interest for an old polymer. *Plant Sci.*, **180**, 399–413.

Razeq, F.M. (2013) *Screening for transcriptional regulators of suberin biosynthesis in plants.*

Riechmann, J.L., Heard, J., Martin, G., et al. (2000) Arabidopsis Transcription Factors: Genome-Wide Comparative Analysis Among Eukaryotes. *Science.*, **290**, 2105–2110.

Rowland, O. and Domergue, F. (2012) Plant fatty acyl reductases: Enzymes generating fatty alcohols for protective layers with potential for industrial applications. *Plant Sci.*

Schmidt, T., Samaras, P., Frejno, M., et al. (2018) ProteomicsDB. *Nucleic Acids Res.*, **46**, D1271–D1281.

- Schreiber, L., Franke, R. and Hartmann, K.** (2005) Wax and suberin development of native and wound periderm of potato (*Solanum tuberosum* L.) and its relation to peridermal transpiration. *Planta*, **220**, 520–530.
- Schütze, K., Harter, K. and Chaban, C.** (2009) Bimolecular fluorescence complementation (BiFC) to study protein-protein interactions in living plant cells. *Methods Mol. Biol.*, **479**, 189–202.
- Schwinn, K.E., Boase, M.R., Marie Bradley, J., Lewis, D.H., Deroles, S.C., Martin, C.R. and Davies, K.M.** (2014) MYB and bHLH transcription factor transgenes increase anthocyanin pigmentation in petunia and lisianthus plants, and the petunia phenotypes are strongly enhanced under field conditions. *Front. Plant Sci.*, **5**, 1–11.
- Sirko, A., Wawrzynska, A., Rodriguez, M.C. and Sektas, P.** (2015) The family of LSU-like proteins. *Front. Plant Sci.*, **5**, 1–9.
- To, A., Joubès, J., Thueux, J., Kazaz, S., Lepiniec, L. and Baud, S.** (2020) AtMYB92 enhances fatty acid synthesis and suberin deposition in leaves of *Nicotiana benthamiana*. *Plant J.*, 1–17.
- Urao, T., Yamaguchi-Shinozaki, K., Urao, S. and Shinozaki, K.** (1993) An Arabidopsis myb Homolog Is Induced by Dehydration Stress and Its Gene Product Binds to the Conserved MYB Recognition Sequence. *Plant Cell*, **5**, 1529–1539.
- Verma, P. and Majee, M.** (2013) Seed Germination and Viability Test in Tetrazolium (TZ) Assay. *BIO-PROTOCOL*, **3**.
- Vishwanath, S.J., Delude, C., Domergue, F. and Rowland, O.** (2015) Suberin: biosynthesis, regulation, and polymer assembly of a protective extracellular barrier. *Plant Cell Rep.*, **34**, 573–586.
- Vishwanath, S.J., Kosma, D.K., Pulsifer, I.P., et al.** (2013) Suberin-Associated Fatty Alcohols in Arabidopsis: Distributions in Roots and Contributions to Seed Coat Barrier

- Properties. *PLANT Physiol.*, **163**, 1118–1132.
- Wang, P., Wang, H., Wang, Y., Ren, F. and Liu, W.** (2018) Analysis of bHLH genes from foxtail millet (*Setaria italica*) and their potential relevance to drought stress. *PLoS One*, **13**, 1–16.
- Wang, W.-S., Zhu, J. and Lu, Y.-T.** (2014) Overexpression of AtbHLH112 suppresses lateral root emergence in *Arabidopsis*. *Funct. Plant Biol.*, **41**, 342.
- Xing, S., Wallmeroth, N., Berendzen, K.W. and Grefen, C.** (2016) Techniques for the analysis of protein-protein interactions in vivo. *Plant Physiol.*, **171**, 727–758.
- Xu, C. and Min, J.** (2011) Structure and function of WD40 domain proteins. *Protein Cell*, **2**, 202–214.
- Yadav, V., Molina, I., Ranathunge, K., Castillo, I.Q., Rothstein, S.J. and Reed, J.W.** (2014) ABCG Transporters Are Required for Suberin and Pollen Wall Extracellular Barriers in *Arabidopsis*. *Plant Cell*, **26**, 3569–3588.
- Yeats, T.H., Martin, L.B.B., Viart, H.M., et al.** (2013) The identification of cutin synthase: formation of the plant polyester cutin. *Nat. Chem. Biol.*, **8**, 609–611.
- Zhang, F., Gonzalez, A., Zhao, M., Payne, C.T. and Lloyd, A.** (2003) A network of redundant bHLH proteins functions in all TTG1-dependent pathways of *Arabidopsis*. *Development*, **130**, 4859–4869.
- Zhang, X., Henriques, R., Lin, S.S., Niu, Q.W. and Chua, N.H.** (2006) *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat. Protoc.*, **1**, 641–646.
- Zhao, H., Wang, X., Zhu, D., Cui, S., Li, X., Cao, Y. and Ma, L.** (2012) A single amino acid substitution in IIIf subfamily of basic helix-loop-helix transcription factor AtMYC1 leads to trichome and root hair patterning defects by abolishing its interaction with partner proteins in *Arabidopsis*. *J. Biol. Chem.*, **287**, 14109–14121.

Zhao, L., Gao, L., Wang, H., Chen, X., Wang, Y., Yang, H., Wei, C., Wan, X. and Xia, T.

(2013) The R2R3-MYB, bHLH, WD40, and related transcription factors in flavonoid biosynthesis. *Funct. Integr. Genomics*, **13**, 75–98.

Zhou, J.J., Liang, Y., Niu, Q.K., Chen, L.Q., Zhang, X.Q. and Ye, D. (2013) The Arabidopsis general transcription factor TFIIIB1 (AtTFIIIB1) is required for pollen tube growth and endosperm development. *J. Exp. Bot.*, **64**, 2205–2218.

Zimmermann, I.M., Heim, M.A., Weisshaar, B. and Uhrig, J.F. (2004) Comprehensive identification of Arabidopsis thaliana MYB transcription factors interacting with R/B-like BHLH proteins. *Plant J.*, **40**, 22–34.

APPENDIX

Table A1: cDNAs and T-DNA mutant lines obtained from the Arabidopsis Biological Resource Center (ABRC). The T-DNA insert location was verified by sequencing.

Gene/protein name (TAIR ID no.)	cDNA (Rowland lab glycerol stock no.)	T-DNA line	T-DNA insert position (relative to +1 of start codon)
bHLH27 (AT4G29930.3)	PYAT4G29930 (OR 1755)	SAIL_104_C12 (CS805006)	Exon 4 (1182 bp)
bHLH111 (AT1G31050.1)	Isolated from root mRNA (OR 805)	1) SALK_065747 2) SALK_001970 3) SAIL_128_D01 (CS806227)	1) Exon 2 (3798 bp) 2) Exon 2 (3602 bp) 3) Exon 2 (3561 bp)
bHLH112 (AT1G61660.1)	G12504 (OR 1752)	SALK_148540	Exon 2 (513 bp)
DWA3 (WD40 protein) (AT1G61210.1)		SALK_046984	Exon 10 (3127 bp)
AT3G50390 (WD40 protein) (AT3G50390.1)	U87735 (OR 1754)	1) SAIL_513_C01 (CS821641) 2) SAIL_255_C01 (CS811949)	1) Exon 1 (201 bp) 2) Exon 1 (651 bp)

Table A2: Primers used for genotyping T-DNA mutant lines.

Gene/protein name (TAIR ID #)	T-DNA line	Primer sequence (5' → 3')
bHLH27 (AT4G29930.3)	SAIL_104_C12 (CS805006)	<i>LP</i> : AAAGAGACAAACGTTGAACCG
		<i>RP</i> : GGATGGGAGAGAAGACGGTAG
bHLH111 (AT1G31050.1)	1) SALK_065747	<i>LP</i> : AACGAAAGGTCAAATGCAAAC
		<i>RP</i> : ATATTTGCCACAAAATGCTGG
	2) SALK_001970	<i>LP</i> : ACGCTAAGAAACAATCCATGC
		<i>RP</i> : AACGAAAGGTCAAATGCAAAC
	3) SAIL_128_D01 (CS806227)	<i>LP</i> : ATTATTGGCCACTCGAAGATG
		<i>RP</i> : TGCATGGTAGCATCCTTAAGC
bHLH112 (AT1G61660.1)	SALK_148540	<i>LP</i> : ATCATTCACCTTTCCGAAAGG
		<i>RP</i> : TTCGGATCATTTCTTGTCACC
DWA3 (AT1G61210.1)	SALK_046984	<i>LP</i> : CTCTGGATGATCAGTCCTTCG
		<i>RP</i> : ATTTTGCTTCATCAGTCGGTG
AT3G50390 (AT3G50390.1)	1) SAIL_513_C01 (CS821641)	<i>LP</i> : TTCCCAAAAATTAACGGTTCC
		<i>RP</i> : TATCAAGCAACGGAAAGATGG
	2) SAIL_255_C01 (CS811949)	<i>LP</i> : GTTAAGGTGAACCGGAAGTCC
		<i>RP</i> : AAACGTTTCATCGTCGTAGG

Table A3: Primers (left-border) used for genotyping T-DNA mutant lines.

BP Primer ID	Primer sequence (5' → 3')
SALK LBb1.3	ATTTTGCCGATTCGGAAC
SAIL LB3	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC

Table A4: Primers used for amplification of *bHLH111* gene promoter for insertion into the TOPO-D pENTR plasmid (CACC on 5' end of the forward primer, FP, for directional TOPO cloning)

Construct name	Primer sequence (5' → 3')
Promoter_{bHLH111}:GUS	FP: CACCATATGTGATGTGCAAATTTTAAAC
	RP: TTTCCTATCTTCGAGCTTTTCTTTTG

Table A5 (continued on next page): Primers used for amplification of coding regions to generate constructs for yeast 2-hybrid experiments

Construct name (with corresponding vector)	Primer sequence (5' → 3')
N-MYB92 bait (pGBKT7)	FP: CATGGAGGCCGAATTCATGGGAAGATCTCCTATCTCTG
	RP: GCCGCTGCAGGTCGACCTAAGACGGTTGAAGAAGGTATTGG
N-MYB93 bait (pGBKT7)	FP: CATGGAGGCCGAATTCATGGGGAGGTCGCCTTGT
	RP: GCCGCTGCAGGTCGACCTATTGGAGATATTGTAGATTGGCT
bHLH27 bait (pGBKT7)	FP: AGGAGGACCTGCATATGATGGAAGATCTCGACCATGAG
	RP: GCCGCTGCAGGTCGACTCAAACCAAAACAAGACACGTACAG
bHLH111 bait (pGBKT7)	FP: AGGAGGACCTGCATATGATGTTAAGGGAAGAATGTACTCC
	RP: GCCGCTGCAGGTCGACCTAACGATACAAAGAACCTCTATAC
bHLH112 bait (pGBKT7)	FP: AGGAGGACCTGCATATGATGGCGGAGGAGTTTAAAGCTACG
	RP: GCCGCTGCAGGTCGACTCACCTGAAATTGTTGCCCCC
bHLH27 prey (pGADT7)	FP: CAGATTACGCTCATATGATGGAAGATCTCGACCATGAG
	RP: TCATCTGCAGCTCGAGTCAAACCAAAACAAGACACGTACAG
bHLH111 prey (pGADT7)	FP: CAGATTACGCTCATATGATGTTAAGGGAAGAATGTACTCC
	RP: TCATCTGCAGCTCGAGCTAACGATACAAAGAACCTCTATAC

N-bHLH111 prey (pGADT7)	<i>FP:</i> CAGATTACGCTCATATGATGTTAAGGGAAGAATGTACTCC
	<i>RP:</i> TCATCTGCAGCTCGAGTTATTTGGGTAGTTCCTTCTCTGGC
C-bHLH111 prey (pGADT7)	<i>FP:</i> CAGATTACGCTCATATGGCCAAACTTCGAGACAAGATC
	<i>RP:</i> TCATCTGCAGCTCGAGCTAACGATACAAAGAACCTCTATAC
bHLH112 prey (pGADT7)	<i>FP:</i> CAGATTACGCTCATATGATGGCGGAGGAGTTTAAAGCTACG
	<i>RP:</i> TCATCTGCAGCTCGAGTCACCTGAAATTGTTGCCCCCA
AT3G50390 (WD40 protein) prey (pGADT7)	<i>FP:</i> GGAGGCCAGTGAATTCATGTCTTCAATGCATAAGAAAGGT
	<i>RP:</i> CGAGCTCGATGGATCCCTATTTTTGTGGACTAATCCTCCCT

Table A6: Primers used for amplification of coding regions to generate constructs for yeast 3-hybrid experiments

Construct name (with corresponding vector)	Primer sequence (5' → 3')
N-MYB92 bait (pBridge MCSI)	<i>FP:</i> TGTATCGCCGGAATTCATGGGAAGATCTCCTATCTCTG
	<i>RP:</i> TTGGCTGCAGGTCGACCTAAGACGGTTGAAGAAGGTATTGG
N-MYB93 bait (pBridge MCSI)	<i>FP:</i> TGTATCGCCGGAATTCATGGGGAGGTCGCCTTGT
	<i>RP:</i> TTGGCTGCAGGTCGACCTATTGGAGATATTGTAGATTGGCT
AT3G50390 (WD40 protein) bridge (pBridge MCSII)	<i>FP:</i> AGAAAGGTGGCGCCGCATGTCTTCAATGCATAAGAAAGGT
	<i>RP:</i> ATCAGCCCGAAGATCTCTATTTTTGTGGACTAATCCTCCCT

Figure A1: Schematic diagram of the pBridge vector (catalog number 630404, Takara Bio). ADH1, alcohol dehydrogenase (promoter and terminator sequences); MCS1, multiple cloning site 1 for 'bait' coding sequence; MCS2, multiple cloning site 2 for 'bridge' coding sequence (the resulting conditionally expressed from the *MET25* promoter in response to methionine levels in the medium, repressed in the presence of 1 mM methionine and expressed in the absence of methionine); HA, hemagglutinin; SV40 NLS, simian virus 40 nuclear localization sequence; PGK1,

phosphoglycerate kinase (terminator sequence); ori, Col E1 origin of replication.

The auxotrophic marker TRP1 used for selection in yeast and ampicillin used for selection in bacteria.

Figure A2: Enzymatic reaction of β -glucuronidase (GUS) reaction used for promoter : reporter gene assays. Conversion of colourless X-Gluc substrate by β -glucuronidase (GUS), which is encoded by bacterial *uidA* gene, to a chloro-bromoindigo product that dimerizes, turns blue and precipitates upon oxidation provides the principle for the assay. Plants lack this enzyme.

Figure A3: Chemistry of tetrazolium red (TZ) assay used for seed viability staining. Within living tissues, dehydrogenases involved in cellular respiration use an electron donor such as NAD(P)H to reduce triphenyl tetrazolium chloride (TTC) into triphenylformazan (TPF), a stable red precipitate.

SUPPLEMENTARY DATA

Figure S1: Protein interaction analysis of AtbHLH27, AtbHLH111, and AtbHLH112 transcription factors using STRING software (string-db.org). All PPIs were determined by yeast 2-hybrid experiments (Jin *et al.*, 2011)(Brady *et al.*, 2011)(Arabidopsis Interactome Mapping Consortium (AIMC), 2011).

Testing MYB proteins (N-terminal R2R3-containing region) for autoactivation

Figure S2: Autoactivation of Y2H reporter genes (*ADE2*, *HIS3*, *MEL1*) by the N-MYB53 bait construct, in the absence of an interacting prey protein. No autoactivation was demonstrated by N-MYB92 and N-MYB93 bait constructs. The reporter gene *MEL1* encodes the nascent α -galactosidase enzyme in several yeast strains. As a result of two-hybrid interactions (where GAL4-BD and GAL4-AD are in close proximity), α -galactosidase is expressed and secreted by the yeast cells, so that colonies turn blue in the presence of the chromogenic substrate X- α Gal. Data provided by Dr. Sofia Khalil. SD, synthetic dropout media.

Figure S3: Autoactivation of Y2H reporter genes (*ADE2*, *HIS3*, *MEL1*, *AUR1-C*) by the AtbHLH112 bait construct, in the absence of an interacting prey protein. No autoactivation was demonstrated by AtbHLH27 or AtbHLH111 bait constructs (no blue colonies and growth on -adenine and -histidine media). The reporter gene *MEL1* encodes the nascent α -galactosidase enzyme in several yeast strains. As a result of two-hybrid interactions (where GAL4-BD and GAL4-AD are in close proximity), α -galactosidase is expressed and secreted by the yeast cells, so that colonies turn blue in the presence of the chromogenic substrate X- α Gal (X). The reporter gene *AUR1-C* is a dominant mutant version of *AUR1*, which encodes the enzyme inositol phosphoryl ceramide synthase that confers strong resistance to the otherwise highly toxic drug Aureobasidin A (A). TDO, triple dropout media

Table S1 (continued on next page): Suberin monomer composition in roots of wild-type and T-DNA insertion *loss-of-function* mutants (two-week-old *A. thaliana* seedlings grown on tissue culture media). The amounts are µg total suberin monomer content per mg of delipidated dried root tissue (DW = dry weight). Error bars represent standard deviation (SD). Red and blue coloured data represent a statistically significant difference of mutant compared to wild-type (Student's *t*-test; red, $p < 0.05$; blue, $p < 0.01$).

Suberin compounds	Average value of monomer content in depolymerized suberin from root tissue (µg/mg DW)					
	Wild-type (Col-0)	<i>bhlh27-1</i>	<i>bhlh111-2</i>	<i>bhlh112-1</i>	<i>dwa3-1</i>	<i>at3g31050-2</i>
Hydroxycinnamates <i>trans</i> -ferulate	0.18 ± 0.02	0.20 ± 0.01	0.14 ± 0.01	0.18 ± 0.01	0.16 ± 0.01	0.16 ± 0.01
Fatty Acids						
*C16:0	0.33 ± 0.03	0.44 ± 0.08	0.23 ± 0.01	0.33 ± 0.01	0.41 ± 0.04	0.45 ± 0.01
*C18:2	0.19 ± 0.02	0.25 ± 0.03	0.10 ± 0.01	0.23 ± 0.01	0.19 ± 0.01	0.31 ± 0.01
*C18:1	0.28 ± 0.04	0.42 ± 0.09	0.16 ± 0.01	0.40 ± 0.01	0.34 ± 0.03	0.42 ± 0.02
*C18:0	0.10 ± 0.03	0.10 ± 0.02	0.07 ± 0.01	0.10 ± 0.01	0.05 ± 0.01	0.07 ± 0.01
C20:0	0.26 ± 0.02	0.19 ± 0.01	0.26 ± 0.01	0.16 ± 0.01	0.28 ± 0.01	0.28 ± 0.01
C22:0	1.35 ± 0.06	1.35 ± 0.06	1.21 ± 0.01	1.29 ± 0.01	1.53 ± 0.05	1.43 ± 0.02
C24:0	0.37 ± 0.02	0.44 ± 0.02	0.29 ± 0.01	0.43 ± 0.01	0.43 ± 0.02	0.42 ± 0.01
C26:0	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
<i>Total</i>	2.91 ± 0.39	3.24 ± 0.41	2.34 ± 0.38	2.95 ± 0.40	2.87 ± 0.45	3.05 ± 0.41
Omega Hydroxy Fatty Acids						
C16:0	0.54 ± 0.04	0.46 ± 0.02	0.50 ± 0.01	0.39 ± 0.01	0.55 ± 0.03	0.51 ± 0.01
C18:1	3.35 ± 0.22	3.20 ± 0.18	2.98 ± 0.07	2.84 ± 0.08	3.66 ± 0.12	3.43 ± 0.05
C20:0	0.26 ± 0.02	0.20 ± 0.01	0.26 ± 0.01	0.18 ± 0.01	0.28 ± 0.01	0.27 ± 0.01
C22:0	1.22 ± 0.08	1.33 ± 0.06	1.20 ± 0.02	1.25 ± 0.04	1.26 ± 0.06	1.16 ± 0.02
C24:0	0.23 ± 0.02	0.29 ± 0.01	0.20 ± 0.01	0.29 ± 0.01	0.25 ± 0.01	0.25 ± 0.01
<i>Total</i>	5.60 ± 1.17	5.18 ± 1.12	5.15 ± 1.04	4.95 ± 1.00	6.00 ± 1.28	5.62 ± 1.20

Primary Fatty Alcohols						
C18:0	0.45 ± 0.04	0.34 ± 0.01	0.57 ± 0.03	0.36 ± 0.01	0.43 ± 0.02	0.39 ± 0.01
**C20:0	0.43 ± 0.03	0.39 ± 0.04	0.31 ± 0.01	0.33 ± 0.01	0.52 ± 0.03	0.39 ± 0.04
C22:0	0.43 ± 0.04	0.70 ± 0.01	0.42 ± 0.02	0.79 ± 0.04	0.39 ± 0.02	0.34 ± 0.01
<i>Total</i>	<i>1.31 ± 0.03</i>	<i>1.43 ± 0.16</i>	<i>1.30 ± 0.11</i>	<i>1.48 ± 0.21</i>	<i>1.34 ± 0.05</i>	<i>1.12 ± 0.03</i>
Dicarboxylic Fatty Acids						
C16:0	0.53 ± 0.03	0.45 ± 0.01	0.49 ± 0.01	0.42 ± 0.01	0.62 ± 0.03	0.58 ± 0.01
**C18:2	0.43 ± 0.03	0.39 ± 0.05	0.31 ± 0.01	0.33 ± 0.01	0.52 ± 0.03	0.39 ± 0.04
C18:1	1.61 ± 0.08	1.49 ± 0.05	1.34 ± 0.02	1.34 ± 0.03	1.86 ± 0.07	1.84 ± 0.03
C18:0	0.27 ± 0.02	0.20 ± 0.01	0.26 ± 0.01	0.18 ± 0.01	0.29 ± 0.01	0.28 ± 0.01
C20:0	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.09 ± 0.01	0.08 ± 0.01
C22:0	0.13 ± 0.01	0.14 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	0.13 ± 0.01
C24:0	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
<i>Total</i>	<i>3.15 ± 0.50</i>	<i>2.84 ± 0.49</i>	<i>2.69 ± 0.45</i>	<i>2.53 ± 0.45</i>	<i>3.63 ± 0.63</i>	<i>3.41 ± 0.62</i>
Total	13.06 ± 0.74	13.07 ± 0.70	11.62 ± 0.16	12.10 ± 0.24	14.35 ± 0.53	13.59 ± 0.17

*C16 and C18 fatty acids here are assumed to be monomers extracted from the suberin polymer, but there may be possibility of contamination from membrane lipids.

** Acetylated C20:0 primary fatty alcohol and C18:2 DCA derivativized monomers co-eluted from the column of the gas chromatograph.