Transcriptional Regulators of Suberin Biosynthesis in *Arabidopsis thaliana*

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Abstract

Like all vascular plants, the root endodermis of *Arabidopsis thaliana* contains aliphatic suberin, a cell wall-associated hydrophobic polymer that helps direct the symplastic movement of water and solutes into the central vasculature. The enzymes involved in suberin biosynthesis are relatively well characterized, but there are many gaps in our knowledge on how suberin biosynthesis is regulated. In this study, I demonstrate that *Arabidopsis* MYB41, MYB53, MYB92, and MYB93, which are R2R3 MYB-type transcription factors, can activate the transcription of suberin biosynthesis genes through their promoter (region 5' of the translation start site). Using a promoter: reporter deletion series, two regions of the promoter upstream region of the *Arabidopsis* *FAR4* gene, which encodes a suberin biosynthetic enzyme, were determined to be important for specific expression in the root endodermis. AC-rich motifs, which are important for the recognition of DNA by specific MYB-type transcription factors, were enriched in the promoters of suberin biosynthesis genes. Although the important *FAR4* promoter DNA segments contained these AC-rich motifs, I found no evidence of direct binding by MYB53, MYB92, and MYB93 using a yeast 1-hybrid assay. Lastly, the promoter of *Arabidopsis* *WRKY56*, which also encodes a transcription factor, was transcriptionally activated by MYB41, MYB53, MYB92, and MYB93. While *WRKY56* had limited impact on root suberin deposition under non-stress conditions, bioinformatic evidence suggests that *WRKY56* has a role under stress conditions.

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List of Symbols, Nomenclature, and Abbreviations

α – Alpha

β – Beta

g - gram

L - Litre

μ - Micro

m – milli

n - nano

ω – Omega

NAME (all caps, not italics) – Name of wild-type protein

NAME (all caps, italics)– Name of wildtype gene

name (lowercase, italics)– Name of mutant gene

name-# (lowercase, italics) - Name of mutant gene with allele number

AHC – Alkyl Hydroxy-Cinnamate

ABCG – ATP-Binding Cassette transporter

amp-DAP – amplified-DAP

ASFT – Aliphatic Suberin Feruloyl Transferase

BCCP – Biotin Carboxyl Carrier Protein

bHLH - basic Helix-Loop-Helix

CYP - Cytochrome P450

DAP – DNA-Affinity Purification

DNA – Deoxyribonucleic Acid

FAR – Fatty Acyl Reductase

G3P – Glycerol-3-Phosphate

gDNA – genomic DNA

GD_{SL} – Glycine (G) Asparagine (D) Serine (S) Leucine (L)

GELP – GD_{SL}-like Esterase and Lipase

GPAT – Glycerol-3-Phosphate Acyl Transferase

GUS – β -Glucuronidase

KCS – β -Ketoacyl-CoA Synthase

LiAc – Lithium Acetate

LACS – Long-chain Acyl-CoA Synthetase

MBW – MYB-bHLH-WD40

MS – Murashige and Skoog

MYB – Myeoblastosis

mRNA – mature Ribonucleic Acid

NAC – NAM (NO APICAL MERISTEM) ATAF (ARABIDOPSIS THALIANA ACTIVATING FACTOR) CUC (CUP-SHAPED COTYLEDON)

PCR – Polymerase Chain Reaction

RT – Room Temperature

PEG-3350 – Poly-Ethylene Glycol (Molecular Weight 3350 g/mol)

pro – promoter or 5' region upstream from translation start site

SHR – Short-Root

ssDNA – single stranded DNA

T-DNA – Transfer-DNA

TEM – Transmission Electron Microscopy

TF – Transcription Factor

UV - Ultraviolet

WRKY – Tryptophan (W) Arginine (R) Lysine (K) Tyrosine (Y)

WT – Wild-Type

Y1H – Yeast-One(1)-Hybrid

YPAD – Yeast Peptone Adenine-sulphate Dextrose

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1- Introduction

1.1- General opening statement

Vascular plants, or *Tracheophyta*, represent the overwhelming majority of known plant species. They are present in our forests (e.g. *Acer saccharinum* - silver maple), our crop fields (e.g. *Triticum aestivum* - bread wheat), and our gardens (e.g. *Petunia axillaris* - large white petunia). Understanding how vascular plants function will help researchers protect and improve them. All vascular plants contain central lignified tissues known as xylem, which transport water and nutrients from the roots to every other part of the plant, and phloem, which moves sugar from the leaves to non-photosynthesizing parts of the plant. The efficiency of this central bundle of conductive tissue that stretches throughout the entire plant, called the stele, has enabled vascular plants to be more widely distributed than non-vascular plants, or *Bryophyta*. In the roots of *Tracheophyta*, surrounding the stele is the endodermis, a single layer of cells whose walls are embedded with a hydrophobic material called suberin that helps maintain the symplastic movement of water and nutrients into the stele (Baxter *et al.*, 2009; Nawrath *et al.*, 2013). In this study, I investigated the regulation of suberin deposition in the root endodermis through the action of transcriptional regulators.

1.2- *Arabidopsis thaliana*: a model system for the study of roots in vascular plants

Taxonomically, vascular plants are initially separated into two groups. There are vascular plants that produce seeds (*Spermatophytina*) and vascular plants that do not produce seeds (*Polypodiophytina* and *Lycopodiophytina*). The *Spermatophytina* are divided into plants that bear naked seeds (*Gymnosperms*: *Cycadopsida*, *Ginkgoopsida*, *Pinopsida*, and *Gnetopsida*) and plants that bear seeds within fruit (*Angiosperms* or *Magnoliopsida*). *Angiosperms* are made up of two groups: the monocotyledons (monocots) and eudicotyledons (eudicots), which together comprise most of the vascular plants that are valued for human health, whether it be for food or medicine (Raskin *et al.*, 2002).

Arabidopsis thaliana (L.) Heynh. is a ubiquitously used plant model organism because it has a short life-cycle, mass seed amplification, a relatively small and diploid genome, and *Agrobacterium*-mediated transformation has made it easy to produce reporter, over-expression, and knockout mutant strains (Koornneef & Meinke, 2010). *A. thaliana* is also a suitable model for studying root hydrophobic barriers in *Angiosperms* and all vascular plants. Roots are not the reason for the evolutionary divergences in vascular plants until the *Angiosperms* are divided into eudicots and monocots, so it is reasonable to believe root anatomy and function does not vary significantly across most vascular plants. Monocots usually have an ephemeral root that can be derived from the stems and leaves (adventitious roots) or the mesocotyl (seminal roots) and tend to branch in many directions while remaining near the top of the soil. Eudicots usually have a single primary root (tap root) that is derived from the radicle and can grow vertically into the soil with lateral roots branching from the tap root (Atkinson *et al.*, 2014; Seago & Fernando, 2013). The stele in the young roots of eudicots and monocots are encircled by an endodermis that contains the cell wall-associated hydrophobic barriers suberin and lignified Casparian strips (Zeier *et al.*, 1999). The same internal root structure is observed in seedless vascular plants like the *Polypodiophytina* (ferns) (Chapple & Peterson, 1987), vascular plants bearing naked seeds like *Ginkgo biloba* (Bonacorsi & Seago, 2016), vascular plants that bear fruits like the monocot *Zea mays* (maize) (Zeier *et al.*, 1999) and the eudicot *A. thaliana* (Nawrath *et al.*, 2013). Therefore, *A. thaliana* is a good model organism for the study of cell-wall associated hydrophobic barriers in the root endodermis of vascular plants.

1.3- Deposition and functions of extracellular, hydrophobic barriers in Arabidopsis

Cell wall-associated hydrophobic barriers are integral to the survival of land plants, largely due to their ability to control the movement of water. Aerial plant organs (i.e. leaf) are coated with a hydrophobic barrier called the cuticle. The cuticle, which is composed of a glycerolipid polyester called cutin and

associated waxes, is deposited on the outer cell wall of epidermal cells. The location and composition of the cuticle means it has a role in preventing uncontrolled water loss via transpiration, protection from plant pathogens, shielding the plant from UV-light, and protecting against various abiotic stresses (Franke *et al.*, 2005; Nawrath *et al.*, 2013). When wounded, the aerial tissues can deposit another hydrophobic barrier, called suberin, to 'seal' the wound (Vishwanath *et al.*, 2015).

Roots contain two distinct hydrophobic barriers: Casparian strips and aliphatic suberin. Casparian strips, which mark stage I of root endodermis differentiation, are deposited in the primary wall of endodermal cells, and take up the entire intercellular space (apoplast) between adjacent endodermal cells. This creates a ring-like barrier along the root endodermis that prevents the uncontrolled apoplastic movement of water and solutes to the stele (Barberon *et al.*, 2016; Barbosa *et al.*, 2019; Naseer *et al.*, 2012). The Casparian strip (names after its founder Robert Caspary) is made of a lignin-like polymer (Naseer *et al.*, 2012). During stage II of root endodermal cell differentiation, aliphatic suberin is deposited on the inner face of the secondary cell wall as to encase the whole cell (**Figure 1**) (Barberon *et al.*, 2016; Vishwanath *et al.*, 2015). Suberin is a heteropolymer containing glycerol, fatty acids (including omega hydroxy and dicarboxylic fatty acids), fatty alcohols, and phenolic compounds (Vishwanath *et al.*, 2015). In the young root's endodermis, the localization and composition of suberin allows it to regulate the transcellular movement of water and solutes (Barberon *et al.*, 2016; Baxter *et al.*, 2009). Together, Casparian strips and aliphatic suberin direct water and solute flow to the symplastic pathway to the xylem of the roots (Enstone *et al.*, 2002; Nawrath *et al.*, 2012). Unlike Casparian strips, aliphatic suberin is also deposited at other tissue-tissue or plant-environment interfaces, such as the root exodermis, root and tuber periderms, leaf bundle sheath cells, and the seed coat (Barbosa *et al.*, 2019; Vishwanath *et al.*, 2015).

Figure 1: Schematic diagram of a cross-section of *A. thaliana* roots during their primary growth stage (left) and after their secondary growth stage (right). Gold ovals represent Casparian Strips and red represents suberin encasing endodermis cells (primary growth stage) and suberized cells of the periderm (secondary growth stage).

1.4- Structure and composition of suberin

Under transmission electron microscopy (TEM), suberin has a lamellae structure with an alternating light/dark banding pattern, indicating that suberin is not just a single layer, but a multilayered polymer.

It has been proposed that these alternating light and dark bands represent distinct polyaliphatic and polyphenolic domains, respectively, but this has not been proven (Franke & Schreiber, 2007; Graça, 2015; Schreiber, 2010). Some have suggested a simpler definition for suberin: a glycerolipid polyester with minimal phenolic content (i.e. ferulate) that can be closely associated with a phenolic/lignin-like polymer, for example the Casparian strip (Geldner, 2013).

The polyaliphatic nature of suberin is due to the presence of C16 – C24 ω -hydroxy fatty acids, α , ω – dicarboxylic fatty acids, mid-chain oxygenated fatty acids, unsubstituted fatty acids, primary fatty alcohols, and α , ω -diols (**Figure 2**). Glycerol is a major component of suberin and was initially thought to

link components of the polyaliphatic domain to the polyphenolic domain via ester linkages (Franke & Schreiber, 2007; Graça, 2015; Vishwanath *et al.*, 2015). However, glycerol is more likely integrated into the aliphatic suberin polymer to potentially form extensive branching of the polymer, as glycerol does in cutin (Geldner, 2013; Yang *et al.*, 2016). Ferulate has also been shown to be a component of suberin's polyaliphatic domain. Non-covalently linked waxes, including alkyl hydroxycinnamates (AHC's), have also been reported to be associated with the suberin polymer and may confer 'sealing' properties (Delude *et al.*, 2016; Gou *et al.*, 2009; Molina *et al.*, 2009; Vishwanath *et al.*, 2013). Bearing in mind the debates in definition, the rest of this study will view suberin as an aliphatic polyester that contains some phenolic compounds.

Figure 2: Structure and composition of the suberin polymer. A) TEM image demonstrating the multiple layers and alternating dark/light bands of the suberin polymer (De Silva 2018). B) Hypothesized organizational structure of the suberin polymer (Bernards, 2002). C) Class of monomeric constituents that typically compromise the root suberin polymer.

1.5- Suberin biosynthesis, transport, and assembly

Suberin is composed of various long-chain (C16 and C18) and very-long-chain (C20-C24) fatty acid derivatives, meaning various enzymes from different families are required for its production (**Figure 3**) (Vishwanath *et al.*, 2015). Suberin biosynthesis starts with 16:0, 18:0, and 18:1 fatty acids that are produced *de novo* in the plastid by the fatty acid synthase (FAS) cycle. These specific fatty acids are converted to fatty acyl thioesters linked with Coenzyme-A, either at the plastid outer envelope or the endoplasmic reticulum (ER), via the action of long-chain acyl-CoA synthetase (LACS) enzymes. In *Arabidopsis*, LACS enzymes are known to 'activate' lipid metabolism by generating fatty acyl-CoAs that are subsequently used in cutin and wax biosynthesis, amongst other processes (Shockey *et al.*, 2002; Zhao *et al.*, 2019). Originally found to only be involved in cutin monomer biosynthesis (Bessire *et al.*, 2007; Schnurr *et al.*, 2004), the *lacs2* mutant was recently found to reduce the total suberin polymer in the root endodermis by almost half (Ayaz *et al.*, 2021). In terms of suberin biosynthesis, the LACS-generated fatty acyl thioesters at the ER have three possible fates: the acyl elongation pathway, the acyl reduction pathway, or the acyl oxidation pathway (Vishwanath *et al.*, 2015).

Elongation of fatty acyl-CoAs occurs through the fatty acid elongase (FAE) complex located in the ER. The FAE complex is made up of four enzymes and the first enzyme in this complex, β -ketoacyl-CoA synthase (KCS), condenses malonyl-CoA with an acyl-CoA of a specific chain length to generate an acyl chain 2 carbons longer (Franke & Schreiber, 2007; Vishwanath *et al.*, 2015). For example, *Arabidopsis* KCS2/DAISY and KCS20 are enzymes that elongate 20:0 (very-long-chain) fatty acyl-CoA to 22:0 fatty acyl-CoA (Franke *et al.*, 2009; Lee *et al.*, 2009) and KCS9 catalyzes the elongation of 22:0 fatty acyl-CoA to 24:0 fatty acyl CoA (Kim *et al.*, 2013). Although these KCS enzymes affect suberin biosynthesis, they may elongate fatty acyl-CoAs for other aspects of lipid metabolism. The remaining three enzymes in the FAE complex modify the product of the condensation reaction to regenerate fully reduced acyl-CoAs (Kim *et al.*, 2013). The elongated (C20-C26) fatty acyl CoAs are either incorporated directly into the

suberin polymer, modified through the acyl oxidation pathway, or modified through the acyl reduction pathways (Vishwanath *et al.*, 2015).

The acyl reduction pathway produces fatty primary alcohols from fatty acyl thioester-CoAs through a two-step NADPH-dependent reaction performed by a single FAR enzyme. *Arabidopsis* contains eight FAR enzymes but only three, FAR1, FAR4, and FAR5, are expressed at sites of suberin deposition (i.e. root endodermis, leaf wounds, in response to abiotic stress) as revealed by qPCR and promoter: GUS experiments (Domergue *et al.*, 2010). Fatty alcohols with chain lengths of 18, 20, and 22 carbons typically exists in the suberin polymer. Genetic experiments using *far1*, *far4*, and *far5* single, double, and triple mutants revealed preferences of FAR1 for C22:0-OH, FAR4 for C20:0-OH, and FAR5, for C18:0-OH, with FAR4 also contributing to C22:0-OH production (Domergue *et al.*, 2010; Vishwanath *et al.*, 2013). Transgenic expression experiments in yeast confirmed the FAR chain-length preferences, while showing each FAR is capable of using substrates of each chain-length (Delude *et al.*, 2016; Domergue *et al.*, 2010; Vishwanath *et al.*, 2013). While ~20 % of fatty alcohols produced by FAR1, FAR4, and FAR5 can then be incorporated into the root suberin polymer, ~80 % of root fatty alcohols are found in soluble waxes thought to be associated, not covalently linked, with the suberin polymer (Delude *et al.*, 2016; Vishwanath *et al.*, 2013). Interestingly, the *far1 far4 far5* mutant did not show any alterations to the root suberin lamellae or have root physiological affects, but the seed coat showed an increased permeability of tetrazolium salt and an increased sensitivity to abscisic acid (Delude *et al.*, 2016; Vishwanath *et al.*, 2013).

The acyl oxidation pathway converts fatty acyl chains from 16 to 24 carbons in length into ω -hydroxy fatty acids and α , ω -dicarboxylic fatty acids. Analysis of root suberin composition in *Arabidopsis* at three different root stages revealed ω -hydroxy fatty acids (37-52 %) and α , ω -dicarboxylic acids (28-31 %) were the most prominent components and the percentage of ω -hydroxy acids and α , ω - dicarboxylic acids increases with age (Delude *et al.*, 2016; Höfer *et al.*, 2008). Suberin-associated cytochrome P450

(CYP) monooxygenases from *Arabidopsis* were first characterized via heterologous expression in yeast, where *AtCYP86A1* was shown to act as an ω -hydroxylase on saturated fatty acids ranging from 12 to 18 carbons in length (Benveniste *et al.*, 2006). CYP86A1 is localized to the ER and is specifically expressed in the root endodermis (Höfer *et al.*, 2008). More importantly, a T-DNA knockout of the *CYP86A1* gene revealed a 60 % reduction in total suberin polymer compared to wild-type, led by the loss of 16:0 and 18:0 ω -hydroxy acids and α , ω -dicarboxylic acids (Höfer *et al.*, 2008). CYP86B1 is another ω -hydroxylase localized to the ER and highly expressed in the root endodermis (Compagnon *et al.*, 2009). The T-DNA *cyp86b1* loss-of-function mutants have strong and specific reductions in C22:0 and C24:0 ω -hydroxy acids and α , ω -dicarboxylic fatty acids (Compagnon *et al.*, 2009; Molina *et al.*, 2009). TEM analysis of *cyp86b1* revealed a disturbance to the suberin lamellar structure in root periderm, furthering demonstrating the involvement of CYP86B1 in suberin biosynthesis and the importance of ω -hydroxy acids and α , ω -dicarboxylic fatty acids to integrity of the suberin polymer (Compagnon *et al.*, 2009). Although CYP86A1 hydroxylates the ω -position of 16:0 and 18:0 fatty acids and CYP86B1 acts identically on 22:0 and 24:0 fatty acids, it is not clear if they also then produce the α , ω -dicarboxylic fatty acids with a subsequent oxidation step. The decrease of α , ω -dicarboxylic fatty acids in the *cyp86a1* and *cyp86b1* loss-of-function mutants could be attributed to another unknown enzyme that converts ω -hydroxy fatty acids to α , ω -dicarboxylic fatty acids. If this were the case, then the decrease of α , ω -dicarboxylic fatty acids in the *cyp86a1* and *cyp86b1* mutants would be attributed to the pool of the precursors (ω -hydroxy fatty acids) being depleted (Compagnon *et al.*, 2009; Höfer *et al.*, 2008).

Two other CYP genes, *CYP94B1* and *CYP94B3*, are also involved in suberin biosynthesis (Krishnamurthy *et al.*, 2020; Krishnamurthy *et al.*, 2021). Under non-stress conditions *CYP94B1* was expressed throughout the entire *Arabidopsis* plant. Under salt stress a 4-fold increase was observed in the roots, with a specific localization to the endodermis. *CYP94B1* overexpression, complementation (with *Arabidopsis officinalis CYP94B1*), and mutant experiments demonstrated a role for *CYP94B1* in providing

ω -hydroxy fatty acid (C16 and C22) and α,ω -dicarboxylic fatty acids (C16) that are important for the diffusion barrier properties of the suberin polymer, and ultimately has a positive role in *Arabidopsis*' response to salt-stress (Krishnamurthy *et al.*, 2020). The same role was found for *CYP94B3* (Krishnamurthy *et al.*, 2021). It appears that these two *CYP94* genes complement the *CYP86* suberin biosynthesis genes in the root endodermis to enhance suberin deposition under salt-stress, and it is possible that this same concept can be applied to the other genes involved in suberin biosynthesis.

Partial root suberin depolymerization experiments led to the discovery that the suberin polymer contains fatty acids and their derivatives (ω -hydroxy fatty acids and α,ω -dicarboxylic fatty acids) linked to glycerol through ester bonds. A family of eight *GPAT* genes in *Arabidopsis* are a land plant-specific adaption of GPATs that are unique for building extracellular lipid polymers like cutin and suberin (Graça & Santos, 2006; Yang *et al.*, 2012b). *GPAT5* is primarily expressed in all cell layers of the roots, along with flowers and seeds. A *gpat5* knockout resulted in a 20 – 50 % reduction in suberin-associated 20:0 – 24:0 fatty acids and their derivatives in 1-week-old *Arabidopsis* roots (Beisson *et al.*, 2007). When *GPAT5* was ectopically expressed in yeast and wheat (germ cell-free) translation systems, there was a preference for substrates greater than 20 carbons in length, with no discrimination to oxidation state (i.e. fatty acid, ω -hydroxy fatty acid, or α,ω -dicarboxylic fatty acid), which agrees with the *in vivo* knockout results of *gpat5* mutants. The most closely related enzyme to *GPAT5* is *GPAT7*, and although it is not expressed in roots it is expressed in response to leaf wounding. Over-expression of *GPAT7* resulted in the production of monoacylglycerols containing 22:0 and 24:0 fatty acids and derivatives, indicating it has the same substrate specificity as *GPAT5* but has a specialized role in suberin production following wounding and likely acts in response to other stresses (Yang *et al.*, 2012).

GPAT5 and *GPAT7*, along with most of the *Arabidopsis* GPATs, show a preference for the *sn*-2 positions of glycerol-3-phosphate (G3P) over the *sn*-1 position that GPATs found in other kingdoms have only been known to use (Yang *et al.*, 2012). The ability to transfer acyl-CoA compounds to the *sn*-1 and *sn*-2

positions of G3P is in-line with partial depolymerization experiments done on cork suberin, which revealed the presence of diacylglycerol fragments (Graça & Santos, 2006). All *Arabidopsis* GPATs have a phosphatase domain, with GPAT4 and GPAT6 having phosphatase activity and forms monoacylglycerols (MAG) from G3P and acyl-CoA substrates in yeast and wheat (germ cell-free) translation systems (Yang *et al.*, 2012). Interestingly, GPAT5 has mutations at important phosphatase catalytic residues and can only form lysophosphatidic acid (LPA), which is an intermediate to MAGs (Yang *et al.*, 2012). Why GPAT5 lacks phosphatase activity and what could perform this necessary step in place of GPAT5 has not yet been investigated, while GPAT7 was not tested for phosphatase activity. Fatty alcohols are a part of the suberin polymer, but the amount of fatty alcohols in suberin is not affected by GPAT5 or GPAT7 overexpression or in knockout lines. The use of fatty alcohols as substrates by GPATs were not investigated in the yeast and wheat (germ cell-free) translation systems (Yang *et al.*, 2012). Currently, the enzyme that links fatty alcohols into the suberin polymer is unknown.

The lipids that form cuticle (cutin and waxes) in *Arabidopsis* are transported, at least in part, through the secretory pathway from the ER to the plasma membrane (PM). The transport mechanism for suberin-based lipids produced in the ER is probably similar to that of cuticle-based lipids considering the chemical similarities (McFarlane *et al.*, 2014). Transfer of these suberin-based lipids from the PM to the cell wall are thought to be catalyzed by ATP-binding cassettes (ABC) transporters, which are localized in the PM. Single mutants of *abcg2*, *abcg6*, and *abcg20* did not have a suberin phenotype, while triple mutants of these three ABC transporter genes result in seed coat and root suberin lamellae having perturbed structures, compositions, and properties (Yadav *et al.*, 2014). GPI-anchored nonspecific lipid transfer proteins (nsLTP), like AtLTP1-4, transfer long-chain fatty acids and very-long chain fatty acids from the plasma membrane to the cell wall (Deeken *et al.*, 2016; Edstam *et al.*, 2013). Although nsLTPs have a broad range of substrates, their expression in specific cell types partly dictates what they can transport and it is possible they facilitate the transport suberin precursors.

Once at the inner-face of the cell wall, the suberin-based lipids must be polymerized... Ursache *et al.* (2021) discovered five GDSL-type esterases/lipases (GELPs) that are required for suberin production in the root endodermis. A quintuple mutant of the five GELPs (*gelp22, 38, 49, 51* and *96*) lacks suberin in the root endodermis. These five GELPs are also repressed by auxin, which initiates lateral root growth, as suberin needs to be depolymerized to make way for the lateral root. A separate set of five GELPs (GELP12, 55, 72, 73 and 81) are induced by auxin and subsequently required for suberin depolymerization during lateral root formation (Ursache *et al.*, 2021). Biochemical studies need to be done to prove the enzymatic activity of the ten GELPs and understand the large family of GELP enzymes.

Outside of aliphatic compounds, phenolic compounds, especially hydroxycinnamates (from the phenylpropanoid pathway), are also incorporated into the suberin polymer. The BAHD family of enzymes, which are specific to land plants, are acyl-CoA dependent acyltransferases that have the unique function of connecting cinnamate-CoA thioesters to an acyl chain, like an ω -hydroxy fatty acid (Gou *et al.*, 2009). An *aliphatic suberin feruloyl transferase (asft)* T-DNA knockout mutant showed a specific reduction of ferulate in the root and seed coat suberin polymer of *Arabidopsis* and had no effect on suberin-associated waxes (Molina *et al.*, 2009a). Growth and development sensitivities for *asft* were found when grown in media containing higher than normal levels of salt, revealing a role for ferulate in controlling ion movement as part of the suberin polymer (Gou *et al.*, 2009). Fatty Alcohol:Caffeoyl-CoA Caffeoyl Transferase (FACT), was found to add suberin-acyl chains to caffeate-CoA specifically and *fact* mutants clearly decreased alkyl hydroxy caffeate content in seed coat suberin, root suberin, and root waxes (Kosma *et al.*, 2012). The action of these suberin-associated BAHD family of enzymes likely takes place in the ER, but evidence is not concrete.

Figure 3: Overview of suberin biosynthetic pathway

1.6- Regulators of root suberin deposition

MYB41 is an R2R3-MYB transcription factor (MYB TF) from the model plant *Arabidopsis thaliana* capable of upregulating the synthesis, export, assembly, and cell wall-localized deposition of suberin (Kosma *et al.*, 2014). Only in response to abiotic stress (e.g. high salt) is MYB41 expressed in the root endodermis, and its overexpression corresponds with an increase in the levels of transcripts encoding suberin biosynthetic enzymes (i.e. *CYP86A1*, *CYP86B1*, *GPAT5*, *FAR1*, *FAR4*, *FAR5*, and *ASFT*) (Kosma *et al.*, 2014). AtMYB39 (or 'SUBERMAN') has been shown to promote suberin biosynthesis in the root endodermis, under stress free conditions, using knockout and over-expression lines. Importantly, promoter: reporter gene assays in leaves of *Nicotiana benthamiana* demonstrated that AtMYB39 can transcriptionally activate multiple *Arabidopsis* suberin biosynthetic genes via their promoter (5' region upstream of translation start site) regions (Cohen *et al.*, 2020). AtMYB107 and AtMYB9 are two other R2R3-MYB TFs, highly related to AtMYB39, that regulate the biosynthesis of seed coat suberin and are strongly responsive to osmotic stress in the seed coat (Gou *et al.*, 2017; Lashbrooke *et al.*, 2016). Furthermore, AtMYB107 was found to bind directly to the promoters of suberin biosynthesis genes *FAR1*, *GPAT5*,

FACT, and *CYP86A1* in Y1H and ChIP assays (Gou *et al.*, 2017). In addition to AtMYB39, transcription factors that regulate suberin biosynthesis constitutively (under non-stress conditions) in root endodermis have been discovered in Dr. Rowland's lab: AtMYB53, AtMYB92, and AtMYB93.

MYB53, MYB92, and MYB93 comprise subgroup 24 of the *Arabidopsis* family of R2R3-MYB TFs (Kelemen *et al.*, 2015; Stracke *et al.*, 2001). Through a promoter: reporter gene fusion, it was found that AtMYB53 and AtMYB93 are specifically expressed in the root endodermis, while AtMYB92 is expressed in the root endodermis and root pericycle (Murmu *et al.*, manuscript in preparation, Rowland Lab). All three of these R2R3-MYB TFs have been implicated in positively regulating suberin biosynthesis in the root endodermis under typical development conditions. Overexpression of any one of these R2R3-MYB TFs in leaves of *Nicotiana N. benthamiana* causes ectopic deposition of suberin lamellae (Murmu *et al.*, manuscript in preparation; To *et al.*, 2020). Based on chemical analysis of suberin using AtMYB53/92/93 single, double, and triple mutants, it is suggested that all three R2R3-MYB TFs regulate suberin biosynthesis in the root endodermis in an overlapping fashion (Hu, 2018).

AtMYB93 has been previously indicated as a repressor of lateral root development through its binding to the ARABIDILLO-1 protein, a promoter of lateral root growth (Gibbs *et al.*, 2014). It is possible AtMYB93 has the unique role of inducing suberin deposition in regions where lateral roots are not formed in response to specific developmental and environmental cues. An AtMYB93 homolog, MdMYB93, that was found to be expressed in russeted apple fruit skins and ectopically activates suberin biosynthesis in *N. benthamiana* leaves when over-expressed (Legay *et al.*, 2016). AtMYB92 has been found to bind to the promoter of *BCCP2*, whose encoded protein is part of the acetyl-CoA carboxylate complex that catalyzes the biotin-dependent carboxylation of acetyl-CoA to malonyl-CoA for fatty acid synthesis in plastids (To *et al.*, 2020). Interestingly, AtMYB92 was only capable of binding to and activating the *BCCP2* promoter when two adjacent AC-rich MYB TF binding sites were not mutated. Therefore, there is likely a

need for AtMYB92 to dimerize with itself or another MYB TF for DNA binding to the BCCP2 promoter. Transient transactivation assays also demonstrated that AtMYB92 could transcriptionally activate other promoters in the fatty acid biosynthesis pathway, such as *ACP1* and *LPD1* (To *et al.*, 2020). Further analysis revealed that over-expression of AtMYB92 increased the abundance of suberin biosynthesis mRNA transcripts and suberin deposition, in-line with data found in the Rowland lab ((Murmu *et al.*, manuscript in preparation; To *et al.*, 2020). Therefore, MYB92 may help induce suberin deposition by increasing the pool of fatty acid precursors available to the suberin biosynthetic pathway. To date, no publications have reported a function for AtMYB53. Phylogenetic analysis of the R2R3-MYB TFs reveal subgroup 24, which contains AtMYB53, AtMYB92 and AtMYB93, is closely related to subgroup 11 (containing AtMYB41) and subgroup 10 (AtMYB39, AtMYB107 and AtMYB9) (Kelemen *et al.*, 2015; Stracke *et al.*, 2001). It is therefore possible that R2R3-MYB TF subgroups 28, 10, and 11 all regulate suberin biosynthesis in various plant tissues, some of which are functioning together or in a partially redundant manner. Outside of the R2R3-MYB TF family, NAC046 was shown to promote suberin biosynthesis in the roots of *A. thaliana* when overexpressed (Mahmood *et al.*, 2019), however *NAC046* has also been shown to promote chlorophyll degradation and leaf senescence in *Arabidopsis*, so its connection to root suberin production is in question (Oda-Yamamizo *et al.*, 2016). In *Solanum tuberosum* (Potato), NAC103 repressed the production of suberin-associated waxes in the tuber phellem but had no impact on the suberin polymer (Soler *et al.*, 2020). From the WRKY TF family, WRKY33 and WRKY9 were found to directly upregulate *CYP* genes (*CYP94B1* and *CYP94B3*, respectively) that they co-express with during salt stress. ChIP, Y1H, and promoter: reporter assays demonstrated that WRKY33 directly binds to and activates the *CYP94B1* promoter; and in-turn 16:0 to 22:0 ω -hydroxy fatty acids are increased in the root endodermis (Krishnamurthy *et al.*, 2020). ChIP and EMSA revealed WRKY9 could bind directly to the *CYP94B3* and *CYP86B1* promoters. *CYP94B3* was connected to the production of root endodermis suberin lamellae, more specifically the 20:0 fatty alcohol, 16:0 ω -hydroxy acids, and 16:0 α ,

ω -dicarboxylic acids that make up the suberin polymer (Krishnamurthy *et al.*, 2021). WRKY56 is of interest as it was substantially upregulated in roots over-expressing MYB39 (Cohen *et al.*, 2020) and MYB53, and was downregulated in roots of *myb53 myb92 myb93* triple knockouts (Klein, 2019). Lashbrooke *et al.* (2016) also identified WRKY56 as part of the multi-species gene signature for suberin deposition. Although WRKY33 and WRKY9 were not shown to interact with a R2R3-MYB TF, they have been shown to directly regulate specific genes involved in suberin biosynthesis and it is possible that WRKY56 has a similar role.

1.7- MYB transcription factor interactions

R2R3-MYB TFs have the potential to bind DNA and other proteins. *Arabidopsis* MYB53, MYB92, and MYB93, preferentially bind to synthesized AC-rich MYB TF *cis*-elements in EMSA and/ or Y1H assays (Dubos *et al.*, 2014; Kelemen *et al.*, 2015). However, they have not been shown to bind to the promoters of suberin biosynthetic genes. As described earlier, MYB92 can bind to AC-rich elements in the *BCCP2* promoter, a fatty acid synthesis gene (To *et al.*, 2020). AtMYB39 can transcriptionally activate suberin biosynthetic genes. Y1H assays found evidence of AtMYB39 binding to the *FAR5* ‘promoter’, but none of the nine other suberin biosynthetic gene ‘promoters’ were tested. This leaves the possibility that AtMYB39 indirectly activates suberin biosynthesis (i.e. higher up in a transcriptional cascade) or requires the activity of another transcription factor (i.e. dimerization, like with MYB92 or a non-MYB TF). No other R2R3-MYB TF in subgroups 28, 10, and 11 have been shown to bind to a specific gene promoter.

Most research on protein-protein interactions involving R2R3-MYB TFs has focused on bHLH TF and WD-40 proteins, which form a MBW complex, for example in the context of flavonoid biosynthesis (Amato *et al.*, 2019; Gil-Muñoz *et al.*, 2020; S. Li, 2014). The MBW complex has been mostly studied in relation to proanthocyanin biosynthesis and it allows for increased complexity, diversity, and adaptability of transcriptional regulation. In *Vitis vinifera* (grape vine), WRKY26 enhances the expression of specific

target genes when interacting with the MYB5a/b-MYC1(bHLH TF)-WD40 complex (Amato *et al.*, 2019). There is also evidence of direct interactions between WRKY and MYB TFs in *V. vinifera*, as the stilbene synthase gene was shown to be regulated by different combinations of MYB15 or MYB14 and WRKY3, WRKY24, WRKY43, WRKY52, or WRKY53 (Vannozzi *et al.*, 2018). Although WRKY33 and WRKY9 directly regulate suberin biosynthesis genes alone, it is still possible that they or other WRKY TFs interact with the suberin-associated MYB TFs to regulate transcription of suberin biosynthetic genes.

1.8- Hypothesis

I hypothesize that Arabidopsis MYB41, MYB53, MYB92, and MYB93 directly activate the transcription of suberin biosynthetic genes via binding to the 5' region upstream of the translation start site (the 'promoter'). I predict that each root suberin-associated R2R3-MYB TF will bind to AC-rich *cis*-elements in the promoter of suberin biosynthetic genes. These results will support the redundancies previously observed between *MYB53*, *MYB92*, and *MYB93* single, double, and triple knockout lines (Murmu *et al*, Rowland Lab, manuscript in preparation). Also, I hypothesize that root suberin-associated R2R3-MYB TFs will activate the promoter of WRKY56 and that WRKY56 regulates suberin biosynthesis.

1.9- Experimental Objectives

First, the ability of the root suberin-associated R2R3-MYB TFs to transcriptionally activate the promoter of suberin biosynthesis genes was tested. The TFs were cloned into over-expression vectors, as transcriptional fusions with the 35S promoter, and the promoters were cloned into the pGREENII 0800-LUC reporter vector, as transcriptional fusions with *FIREFLY LUCIFERASE*. The over-expression and reporter vectors were each transformed into *Agrobacterium tumefaciens*, and these cultures were co-infiltrated into the leaves of *N. benthamiana*. Plant cells containing the over-expression and reporter vectors, transferred from the *Agrobacterium*, will produce FIREFLY LUCIFERASE if the TF activates the promoter. Second, a single suberin biosynthesis gene promoter (*FAR4*) was used to determine if and

where root suberin-associated R2R3-MYB TFs bind directly. The minimal size of the *FAR4* promoter required for expression in the root endodermis (where suberin is deposited) was determined. For this, a deletion series of the *FAR4* promoter was made and each deletion was cloned into a reporter gene vector. Multiple stable plant lines expressing each promoter:reporter construct were generated and stained for the expression of the reporter gene. Regions of the *FAR4* promoter identified as important for root endodermis expression were then analyzed using bioinformatics for potential TF binding sites and divided into multiple 100-200 bp fragments. A Y1H assay was used to determine if the root suberin-associated R2R3-MYB TFs could bind to the 100-200 bp fragments of the *FAR4* promoter determined to be important of root endodermis expression. Third, the involvement of WRKY56 in regulating root suberin biosynthesis was characterized using T-DNA insertion mutants.

2- Materials and Methods

2.1- Plant Materials

A. thaliana ecotype Columbia (Col)-0 and *N. benthamiana* ecotype Lab were used in this study. Three T-DNA insertion lines, *wrky56-1*, *wrky56-2*, and *wrky56-3* were obtained from the Arabidopsis Biological Resource Centre (ABRC), Ohio State University (**Supp. Table 1**).

2.2- General Protocols

2.2.1- Seed surface sterilization

Arabidopsis thaliana (Col-0) and *Nicotiana benthamiana* seeds (**Supp. Table 1**) were gently mixed in 70 % (v/v) ethanol for 1 minute and washed with sterile distilled H₂O (dH₂O). Next, the seeds were gently washed with a 1.8 % (v/v) sodium hypochlorite solution for 2 minutes and washed with sterile dH₂O five times, or until there was no smell of the sodium hypochlorite (bleach) solution. After sterilization, all seeds were re-suspended in sterile dH₂O. *A. thaliana* (Col-0) seeds were stratified in the dark at 4 °C for 3 days before being sown. *N. benthamiana* seeds did not undergo stratification.

2.2.2- Growth conditions of *N. benthamiana* infiltrated with *Agrobacterium tumefaciens*

Sterilized *N. benthamiana* seeds were sown into autoclaved soil (Pro-mix BX-General Purpose Growing Medium) that had been water saturated with a fertilizer (fertilizer solution had concentrations of 0.2% (w/v) 20-20-20 macronutrient mix [Plant-Prod] and 0.0125% (w/v) chelated micronutrient mix [Plant-Prod]). Plants were grown under a 16-hr light (180-210 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 22 °C) and 8-hr dark (21 °C) cycle, with the first week of growth under a plastic cover to increase humidity and help promote germination. After the first week, the plant soil was lightly fertilized once a week as described above and additionally watered about twice per week for 5-8 weeks total. Plant watering was stopped three days prior to the infiltration of leaves with *Agrobacterium tumefaciens*.

2.2.3- Growth conditions of *A. thaliana* used for generation of *FAR4* promoter: GUS lines

Sterilized *A. thaliana* (Col-0) seeds were incubated in the dark at 4 °C for three days, then sown into autoclaved soil (Pro-mix BX-General Purpose Growing Medium) that had been water saturated with fertilizer (fertilizer solution had concentrations of 0.2% (w/v) 20-20-20 macronutrient mix [Plant-Prod] and 0.0125% (w/v) chelated micronutrient mix [Plant-Prod]). T₀ plants used for floral dip transformation were grown under a 16-hr light (100-130 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 21 °C) and 8 hr dark (21 °C) cycle. All successive generations were grown under 24 hr of light (100-130 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 21 °C) for seed amplification.

2.2.4- Transformation of chemical competent *E. coli* (DH5 α) cells

Chemical competent *E. coli* (DH5 α) cells (stored at -80 °C) were thawed on ice for 15 minutes. 50 μL of cells were mixed with 10 ng of vector (v) DNA or 2 μL of cloning reaction, and incubated on ice for 15 minutes with gentle mixing every 5 minutes. The cell / DNA mixture was heat shocked for 1 minute at 42 °C, placed on ice for 1 minute, then 450 μL of LB media was added. The transformed cells were incubated at 37 °C for 1 hour with shaking, then centrifuged at 4, 700 x *g* for 1 minute. The supernatant was discarded and the pellet was re-suspended in 100 μL of LB. 90 μL and 10 μL of the resuspended pellet were added separately to LB agar (0.7 %) plates with the appropriate antibiotic (**Supp. Table 2**) and left to incubate overnight at 37 °C, in the dark.

2.2.5- Transformation of electrocompetent *A. tumefaciens* (GV3101::pMP90) cells

Electrocompetent *A. tumefaciens* (GV3101::pMP90) cells (stored at -80 °C) were thawed on ice for 15 minutes. 50 μL of cells were mixed with 50 ng of vector DNA (**Suppl. Table 4**) and incubated on ice for 15 minutes with gentle mixing every 5 minutes. If more than one vector was being transformed into the same cells (i.e. pGREENII 0800-LUC reporters and pSOUP), 50 ng of each vector was used. The cell-DNA mixture was transferred to a pre-chilled electroporation cuvette, and all moisture was wiped off. Electroporation was done at 2.2 kV, 10 μF capacitance, and 630 Ohm resistance on a MicroPulser Electroporator (Bio-Rad). Following electroporation, 450 μL of LB media was immediately added to the

cells. Transformed cells were left to incubate at 30 °C for 2 hours with shaking. The transformed cell culture was centrifuged at 4,700 x *g* for 1 minute, the supernatant discarded, and the pellet was re-suspended in 100 µL of LB. 90 µL and 10 µL of the resuspended pellet were added separately to LB agar (0.7 %) plates with the appropriate antibiotics (**Supp. Table 2**) and left to incubate for 2 days at 30 °C, in the dark.

2.2.6- Preparing and transforming chemically competent yeast cells

A single yeast colony was inoculated in 5 mL of YPAD (or appropriate selective media) and incubated overnight at 30 °C with shaking. The entire yeast culture was added to 45 mL of YPAD (or appropriate selective media), pre-warmed to 30 °C, and left to grow at 30 °C with shaking until the OD_{600nm} of the 50 mL yeast culture doubled twice (~ 8 hours). The 50 mL yeast culture was centrifuged at 1,811 x *g* for 2 minutes. The supernatant was discarded, and the yeast pellet was resuspended in 25 mL sterile dH₂O. The yeast culture was centrifuged at 1,811 x *g* for 2 minutes, the supernatant was discarded, and the yeast pellet was resuspended in 1 mL of 100 mM LiAc (prepared fresh). The 1 mL yeast culture was centrifuged at 6,200 x *g* for 5 seconds, the supernatant was discarded, and the pellet was resuspended in 100 mM LiAc, up to a volume of 500 µL. 50 µL of the now chemically competent yeast culture was added to ~500 ng of DNA and put-on ice. In order, the following were added to each 50 µL chemically competent yeast sample: 240 µL of 50 % (w/v) PEG 3350, 36 µL 1 M LiAc, and 50 µL of 2 mg/mL sheared salmon DNA (single-stranded DNA that was incubated for 10 minutes in boiling water, then put-on ice immediately before adding). Each sample was vortexed for 1 minute, then the samples were heat shocked for 20 minutes at 42 °C. Samples were centrifuged at 6,200 x *g* for five seconds, the supernatant was discarded, and the pellet was resuspended in 1 mL of YPAD media. Samples were then left to grow at 30 °C for 2 hours with shaking, then centrifuged at 6,200 x *g* for five seconds. The supernatant was discarded, and the pellet was re-suspended in 500 µL of sterile dH₂O. The sample was centrifuged at 6,200 x *g* for five seconds, the supernatant was discarded, and the pellet was re-

suspended in 100 μ L of sterile dH₂O. Samples were spread onto Selective Dropout Agar Yeast Media (without specific amino acids or nucleotides; **Suppl. Table 2**) plates and incubated 2-4 days at 30 °C.

2.2.7- Transformation of *E. coli* DH5 α , colony amplification, and vector DNA isolation

For amplification and isolation of vDNA from a single colony after a plasmid transformation or streaking out of a glycerol stock, the following procedure was used. A single *E. coli* colony was used to inoculate 5 mL of LB (25 g/L) with appropriate antibiotic (**Suppl. Table 2**), and then the cells were grown overnight at 37 °C with shaking. The entire 5 mL bacterial culture was harvested through centrifugation and the vDNA was isolated using a High-Speed Plasmid Mini Kit (Geneaid, Cat. # PD300) following the manufacturer's protocol. The quantity and purity of the isolated vDNA was analyzed using a ThermoFisher Nanodrop 1000.

2.2.8- *Arabidopsis thaliana* genomic DNA preparation

About 20 mg of fresh leaf tissue from a young *A. thaliana* (Col-0) plant (2-3 weeks old) was placed in a microcentrifuge tube and ground with an autoclaved pestle, then 400 μ L of Extraction Buffer (200 mM Tris-HCl (pH 7.5), 0.5 % SDS, 25 mM EDTA (pH 8.0), 250 mM NaCl) was added and the leaf tissue was further homogenized. Crude lysates were centrifuged at 16,200 x *g* for 10 minutes (RT) and the genomic DNA (gDNA)-containing supernatant was transferred to a new tube. 350 μ L of isopropanol was mixed with the supernatant to precipitate the gDNA and the mixture was centrifuged at 21,000 x *g* for 10 minutes, and the supernatant was discarded. 800 μ L of 70 % (v/v) ethanol (EtOH) was added to the gDNA pellet to remove excess salts. The solution was centrifuged at 21,000 x *g* for 5 minutes, the supernatant was discarded, and the gDNA pellets were left to air-dry for 30 minutes. Crude gDNA was resuspended in 100 μ L sterile dH₂O and stored at 4 °C until needed.

2.2.9- Gateway cloning reactions

The 4 µL **BP** cloning reaction contained 150 ng of donor vector, 50 ng of attB1-targetDNA-attB2 PCR product, a 1X concentration of GATEWAY® **BP** Clonase™ II Enzyme Mix, and TE buffer (pH 8.0). The **BP** cloning reaction was incubated for 2 hours at 25 °C, then spiked with 1 µg of Proteinase K (ThermoFisher, Cat. # AM2548) and incubated for 10 minutes at 37 °C to stop the reaction. The 5 µL **LR** cloning reaction contained 150 ng of expression vector, 50 ng of donor vector, a 1X concentration of GATEWAY® **LR** Clonase™ II Enzyme Mix, and TE buffer (pH 8.0). The **LR** cloning reaction was incubated for 2 hours at 25 °C, then spiked with 1 µg of Proteinase K (ThermoFisher, Cat. # AM2548) and incubated for 10 minutes at 37 °C. Reaction products were immediately used or stored at -20 °C.

2.2.10- Colony PCR and genotyping PCR

All PCRs contained 2 µL of colony suspension (colony from plate re-suspended in 20 µL of sterile distilled water), or 2 µL of genomic DNA (dissolved in 100 µL of sterile distilled water), and 18 µL of PCR mix (0.25 mM dNTP, 0.3 µM Forward Primer, 0.3 µM Reverse Primer, 0.5 Units Taq Polymerase, and 1X PCR Buffer [20 mM Tris-HCl pH 8.4, 50 mM KCl]). PCRs had 3 stages. Stage 1: 95 °C for 5 minutes. Stage 2: 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1 min./1 Kbp. Stage 2 was repeated 34 more times. Stage 3: 72 °C for 7 minutes.

2.3- Dual-luciferase promoter activation assay via Agrobacterium infiltration of N. benthamiana leaves

2.3.1- Positive controls for dual-luciferase assay

Populus trichocarpa (*Pt*) transcription factors and associated promoter were used as a positive control for the dual-luciferase assay via *Agrobacterium* infiltration of *N. benthamiana* leaves (James *et al.*, 2017). *PtMYB134* and *PtbHLH131* coding sequences were inserted downstream of the 35S promoter in pMDC32 (**Suppl. Figure 6**) and are overexpression plasmids. *PtANR1* promoter was cloned in the *NotI* site of the pGREENII 0800-LUC (luciferase) vector (**Suppl. Figure 3**) and is the reporter plasmid. These vector

constructs were a gift from Dr. Peter Constabel from the University of Victoria, Canada. The two overexpression plasmids were individually transformed, while the reporter plasmid was co-transformed with the pSOUP (**Suppl. Figure 4**) vector into *A. tumefaciens* (GV3101: pMP90) via electroporation ([2.2.5](#)).

2.3.2- Vector construction and strain preparation for promoter: reporter constructs

The pGREENII 0800-LUC (**Suppl. Fig. 2**) reporter vector was obtained from The New Zealand Institute for Plant & Food Research Ltd. The pGREENII 0800-LUC expression cassette, which is flanked by left border (LB) and right border (RB) sites, consists of a renilla luciferase gene under control of a 35S promoter and a firefly luciferase gene downstream of a multiple cloning site (**Suppl. Fig. 2**). The Takara Bio In-Fusion Primer Design Tool was used to design primers (**Suppl. Table 3**) for the amplification of the *FAR4*, *GPAT5*, *CYP86A1*, *CYP86B1*, *ASFT*, and *WRKY56* promoter upstream regions and subsequent insertion between the *Hind*III and *Bam*HI restriction sites of pGREENII 0800-LUC. Upstream regions were amplified from *A. thaliana* (Col-0) genomic DNA ([2.2.8](#)) using iProof™ High-Fidelity DNA Polymerase (Bio-Rad, Cat# 1725330) following the manufacturer's instructions. PCR products were run on a 1 % agarose gel and the desired PCR product was excised from the gel and column purified using the NucleoSpin® Gel and PCR Clean-Up Kit (Takara-Bio, Cat. # 740609.250) following the manufacturer's instructions. The PCR-amplified products were quantified, and purity analyzed using a ThermoFisher Nanodrop 1000.

The pGREENII 0800-LUC vector was transformed into chemically competent DH5α *E. coli* cells ([2.2.4](#)). A single colony was amplified and the pGREENII 0800-LUC reporter vector was isolated and purified ([2.2.7](#)). *Hind*III and *Bam*HI restriction enzymes were used to digest pGREENII 0800-LUC in preparation for cloning. The 20 µL digest contained: 2 µL 10X CutSmart Buffer, 0.5 µL *Hind*III, 0.5 µL *Bam*HI, 1000 ng of pGREEN II 0800-LUC, and topped off with sterile dH₂O. Digests were incubated at 37 °C for 3 hours and digested DNA then purified with the NucleoSpin® Gel and PCR Clean-Up Kit (Takara-Bio, Cat. # 740609.250) following the manufacturer's instructions. The linearized pGREENII 0800-LUC was

quantified on the ThermoFisher Nanodrop 1000. The PCR-amplified upstream regions were individually inserted into the linearized pGREENII 0800-LUC vector with the In-Fusion HD Cloning Kit following the manufacturer's instructions. Two (2) μ L of the completed cloning reaction were immediately transformed into chemically competent *E. coli* (DH5 α) cells ([2.2.4](#)). Colony PCR ([2.2.10](#)) was used to confirm colonies containing successfully DNA constructs, using primers from **Suppl. Table 3**. A single successful colony was amplified and vDNA (pGREENII 0800 *promoter*: LUC) was extracted from the culture ([2.2.7](#)). The insertion was then confirmed via sequencing. pGREENII 0800 *promoter*: LUC constructs were co-transformed with pSOUP into *Agrobacterium tumefaciens* (GV3101::pMP90; [2.2.5](#)).

2.3.3- Vector construction and strain preparation for transcription factor over-expression constructs

The coding sequence for AtMYB39 was amplified from cDNA derived from RNA extracted from the roots of wild-type *Arabidopsis* (Col-0) by Dr. Jhadeswar Murmu. Primers (**Suppl. Table 3**) were designed to amplify the AtMYB39 coding sequence while adding attB1 and attB2 sites to the 5' and 3' end, respectively. PCR products were run on a 1 % agarose gel and the desired PCR product was excised from the gel and column purified using the NucleoSpin® Gel and PCR Clean-Up Kit (Takara-Bio, Cat. # 740609.250) following the manufacturer's instructions. The quantity and purity of the attB1-AtMYB39-attB2 PCR product was analyzed using a ThermoFisher Nanodrop 1000. The attB1-AtMYB39-attB2 amplicon was used to recombine AtMYB39 into pDONR211 (**Suppl. Fig. 4**) via GATEWAY BP cloning ([2.2.9](#)), generating pDONR221:AtMYB39. Two μ L of the completed cloning reaction were immediately transformed into chemically competent *E. coli* (DH5 α) ([2.2.4](#)). Colony PCR was used to confirm colonies containing successfully cloned constructs ([2.2.10](#), primers in **Suppl. Table 3**). A single positive colony was amplified and vDNA (pDONR221:AtMYB39) was extracted and purified ([2.2.7](#)). The LR cloning reaction ([2.2.9](#)) was done with pK7WG2D (**Suppl. Fig. 7**) as the destination vector. Colony PCR was used to confirm colonies containing successfully cloned constructs ([2.2.10](#), primers in **Suppl. Table3**). A single successful colony was amplified and vDNA (pK7WG2D:AtMYB39) was extracted and purified from the culture

([2.2.7](#)). The cloning was then confirmed via Sanger DNA sequencing and pK7WG2D:AtMYB39 was transformed into *Agrobacterium tumefaciens* (GV3101::pmp90, [2.2.5](#)). The Rowland lab already had AtMYB41 (pK7WG2D), AtMYB53 (pK7WG2D), AtMYB92 (pMDC32), AtMYB93 (pVKH18), AtMYB45 (pK7WG2D), and AtMYB67 (pK7WG2D) coding regions in over-expression vectors in *Agrobacterium tumefaciens* (GV3101::pMMP90), as well as pBIN19-p19 in *Agrobacterium tumefaciens* (C58C1::pCH32; **Suppl. Table 2**).

2.3.4- Infiltration of *N. benthamiana* leaves with mixed *Agrobacterium* cultures

Three days before infiltration, glycerol stocks containing *Agrobacterium* strains with the required plasmids (**Suppl. Table 4**) were used to streak out bacteria on LB agar plates with the appropriate antibiotics (**Suppl. Table 2**) and incubated at 30 °C for 2 days in the dark. Two colonies for each *Agrobacterium* strain were inoculated separately into 5 mL of LB with appropriate antibiotics (**Suppl. Table 2**) and grown overnight at 30 °C, with shaking, for 16 to 20 hours. Each infiltration assay contained *Agrobacterium* with pBIN-p19, which encodes the p19 viral suppressor of silencing, an over-expression vector, and a reporter vector.

Each *Agrobacterium* strain was washed three times with 5 mL infiltration buffer (50 mM MES (pH 5.6), 2 mM Na₃PO₄•12H₂O, 0.1 mM acetosyringone, 5 mg/mL glucose) and then re-suspended in 3 mL of infiltration buffer. The infiltration cultures were mixed so that overexpression strains and reporter strains were in a ratio of 10:1, and with overexpression strains in a 1:1 ratio with p19. Each infiltration mixture had a final OD_{600nm} = 0.6. The mixtures were left in the dark for 2 hours at RT. An hour before the infiltration, multiple plants with healthy and similarly sized leaves were removed from the growth chambers and placed under incandescent light. Plants were selected such that six infiltrations could be sufficiently spaced on each leaf. Plants were watered 30 minutes before the infiltration. After a slight puncture with a razor blade to the abaxial epidermal cells, each infiltration culture was injected into the leaf until the infiltration area had a diameter greater than 1 cm. The leaves used were 2-4 leaf positions

away from the apical meristem. The infiltrated leaf area was circled and labelled with marker, and this was done on four separate plants for each infiltration mix ($n = 4$). Infiltrated plants were placed back in the growth chamber and their regular watering/fertilization schedule was continued for 72 hours.

2.3.5- Dual-luciferase assay

Infiltrated plants were removed from the growth chambers after 72 hours. A 0.7 mm-diameter leaf disc was punched out of each infiltrated leaf area and immediately frozen in liquid nitrogen. Leaf tissues were ground to a powder with a sterile pre-chilled pestle (give dimensions or brand) and then warmed to room temperature before 250 μL of 1X Passive Lysis Buffer (PLB) was added to the powdered samples. The samples were re-ground with the same pestle and then incubated for 15 minutes at room temperature to complete the cell lysis. Samples were centrifuged for 20 minutes at $20,817 \times g$ and 4°C , then kept on ice. 5 μL of supernatant was removed to a single well of a white flat-bottom 96-well plate (Nunc). 30 μL of Luciferase Assay Reagent (LAR) II was added and the chemiluminescence was measured. 30 μL of Stop & Glow™ was added and the chemiluminescence was measured again. Luciferase measurements were taken with a five second delay and 10 second integrated measurement on the CYTATION5 (BioTek) plate imaging reader. The ratio of firefly luciferase (suberin gene promoter controlled): renilla luciferase (35S promoter controlled) for each sample was calculated and averaged between biological replicates.

2.4- FAR4 promoter deletion series

2.4.1- Vector construction and bacterial strain preparation

Constructs containing fragments of the *FAR4* promoter (5' upstream from the translation start site) were made by Lena Watkins and Dr. Jhadeswar Murmu, as described below. These regions were PCR-amplified with iProof™ High-Fidelity DNA Polymerase (Bio-Rad, Cat# 1725330) following the manufacturer's instructions, using *A. thaliana* genomic DNA ([2.2.8](#)) as template. For each region, an

attaB1 ‘forward’ primer specific to each deletion and the same attB2 ‘reverse’ primer immediately upstream of the *FAR4* ATG start codon were used (**Supp. Table 3**). The PCR products were run on a 1 % agarose gel and the desired PCR product was excised from the gel (under UV light detection) and column purified using the NucleoSpin® Gel and PCR Clean-Up Kit (Takara-Bio, Cat. # 740609.250) following the manufacturer’s instructions. The attB1-*FAR4*upstreamregion-attB2 PCR products were quantified, and purity analyzed with a ThermoFisher Nanodrop 1000. For each promoter in the deletion series, the attB1-*FAR4*upstreamregion-attB2 construct was recombined into pDONR221 donor vector (**Supp. Figure 4**), via GATEWAY BP cloning ([2.2.9](#)), generating pDONR221:*FAR4*upstream region (**Supp. Table 4**) for each DNA segment in the promoter deletion series. Transformation of the BP cloning reaction, colony PCR confirmation ([2.2.10](#), primers in **Supp. Table 3**), colony amplification, and vDNA extraction were done ([2.2.7](#)). LR Gateway cloning ([2.2.9](#)) was then used to transfer the *FAR4* upstream regions into the expression vector pKGWFS7 (**Supp. Figure 5**; Karimi *et al.*, 2002). Transformation of the LR cloning reaction, colony PCR confirmation (primers in **Supp. Table 3**), colony amplification, and vDNA extraction was done ([2.2.7](#)). The inserts and flanking regions of the plasmid constructs were confirmed via Sanger DNA sequencing. Each pKGWFS7:*FAR4*upstreamregion plasmid in the promoter deletion series was transformed into *A. tumefaciens* (GV3101: pMP90; [2.2.5](#)) and made into glycerol stocks for long-term storage at -80 °C (**Supp. Table 4**).

2.4.2- Floral dip transformation

Wild-type *Arabidopsis* (Col-0) seeds were sterilized, stratified, and grown ([2.2.3](#)). Plants were ready for the floral dip after their second set of inflorescence flowers had bolted, but not opened (5-6 weeks old). Watering was stopped three days before the floral dip. All siliques and opened inflorescence flowers were removed the day of transformation.

Three days before the floral dip transformation, the glycerol stock for each *Agrobacterium* (GV3101: pMP90) strain containing the pKGWFS7: *FAR4*upstreamregion plasmids were streaked on LB agar plates

with the appropriate antibiotics (**Supp. Table 2**) and incubated at 30 °C for 2 days. A single colony for each strain was separately inoculated in 5 mL of liquid LB with appropriate antibiotics (**Supp. Table 2**) and incubated overnight at 30 °C in the dark with shaking. 2.5 mL of the overnight culture were diluted 10X in liquid LB (gentamycin, 40 µg/mL; and spectinomycin, 100 µg/mL) and incubated at 30 °C with shaking for 16 hours. Cultures were centrifuged at 4,700 x *g* for 10 minutes (RT) and the supernatant was removed. Pellets were re-suspended in infiltration buffer (5 % (w/v) sucrose, 0.02 % (v/v) Silwet-77) to an OD_{600nm} = 0.8.

For each *FAR4*pro:GUS construct, two pots containing six *Arabidopsis* (Col-0) WT plants were dipped in each *Agrobacterium* infiltration culture (OD_{600nm} = 0.8.) for 1 minute, so that only the unopened inflorescence flowers were in the culture. Floral dipped plants were placed back in their growth chambers with a plastic cover and wrapped in a black garbage bag. After 24 hours, the plastic cover and garbage bag were removed and the plants grew under normal light conditions until fully developed siliques formed. Seeds from the 12 plants for each *FAR4*pro: GUS constructs were harvested individually and designated the T₁ generation.

2.4.3- Selection, growth, and harvesting of *FAR4*promoter: GUS lines

Harvested T₁ seeds were surface sterilized and stratified as previously described, then ~100 seeds were placed onto an 0.5 % agar plate supplemented with ½ strength Murashige & Skoog (MS) basal medium with Gamborg vitamins, 1 % sucrose, and 50 µg/mL kanamycin. These T₁ seeds were plated along with *A. thaliana* (Col-0) wild-type seeds as the negative control, and kanamycin-resistant seeds previously generated in Dr. Rowland's lab as the positive control. After a week of growth under constant light (100-130 µmol·m⁻²·s⁻¹, 21 °C) on selection agar plates, healthy kanamycin-resistant T₁ seedlings (ones that matched the growth and colour of the positive control) were transplanted to soil and grown for seed amplification as described above. This was done until ~20 individual (independent) T₁ seedlings were selected, amplified, and harvested for their T₂ seeds for each *FAR4*pro: GUS line in the deletion series.

For all independent lines, ~50 T₂ seeds were grown on a 0.5 % agar plate, supplemented with ½ strength Murashige & Skoog (MS) basal medium with Gamborg vitamins, 1 % sucrose, and 50 µg/mL kanamycin for 1 week and the ratio of kanamycin resistant (alive): kanamycin sensitive (dead) seedlings was recorded to identify single loci T-DNA insertion lines (3:1 alive:dead ratio). These seedlings were then genotyped ([2.2.10](#))

2.4.4- GUS staining and light microscopy

Healthy seedlings from the T₂ generation selected using kanamycin were transferred from the selection plate to GUS Staining solution (1 M NaPO₄ (pH 7), 4 mM KFe(CN), 0.1 % (v/v) Triton-x-100, 0.05 % (w/v) X-glucuronidase (dissolved in DMF), and dH₂O) and incubated for 20 hours at 37 °C, in the dark.

Seedlings were soaked in 70 % EtOH for 24 hours at room temperature. This step was repeated if the seedlings still contained chlorophyll. The 70 % EtOH solution was changed, and the stained seedlings were stored indefinitely within sealed glass scintillation vials. Whole plants were placed on a slide, suspended in 25 % glycerol, and placed under a coverslip before being imaged. Bright-field microscopy of GUS activity was observed using a Zeiss AXIO Imager.M2 (compound microscope) with a 10X ocular lens and a 10X objective lens. Images were captured through a Zeiss AxioCam ICc1 camera and analyzed using AxioVision 4.8 software. Automated scale bars of 300 µm were used.

2.5- Bioinformatic prediction of cis-acting elements in the FAR4 promoter upstream region

The *FAR4* promoter (2300 bp 5' from the *FAR4* translation start site) was analyzed for known *cis*-acting elements (consensus DNA motifs; **Table 1**) for MYB, WRKY, bHLH, and NAC transcription factors using PlantPAN3.0 software (<http://plantpan.itps.ncku.edu.tw/promoter.php>), PLACE (<https://www.dna.affrc.go.jp/PLACE/?action=newplace>), and AGRIS (<https://agris-knowledgebase.org/AtcisDB/>) (Chow *et al.*, 2019; Higo *et al.*, 1999; Yilmaz *et al.*, 2011). A manual sequence search was also done for confirmation.

2.6- Yeast 1-hybrid to test direct binding of R2R3-MYB TFs to fragments of the FAR4 promoter

2.6.1- Construction of pBait-AbAi bait vectors

The Takara Bio In-Fusion Primer Design Tool was used to design primers (**Suppl. Table 1**) for the amplification of six different *FAR4* promoter regions, based on the *in silico* *FAR4* promoter analysis, and subsequent insertion between the *Hind*III and *Xho*I restriction sites of the pAbAi vector (**Suppl. Figure 8**). *FAR4* promoter regions were amplified from *A. thaliana* (Col-0) gDNA ([2.2.8](#)) using iProof™ High-Fidelity DNA Polymerase (Bio-Rad, Cat# 1725330), following the manufacturer's instructions and using primers listed in **Suppl. Table 3**. PCR products were column purified using the NucleoSpin® Gel and PCR Clean-Up Kit (Takara-Bio, Cat. # 740609.250) following the manufacturer's instructions. The PCR-amplified *FAR4* upstream region DNA products were analyzed for quantity and purity using a ThermoFisher Nanodrop 1000. The pAbAi vector was transformed into chemically competent DH5α *E. coli* cells, a single colony was amplified, and the pAbAi vector was isolated ([2.2.4](#), [2.2.7](#)). The *Hind*III and *Xho*I restriction enzymes were used to digest pAbAi in preparation for cloning. The 20 µL reaction contained: 2 µL 10X CutSmart Buffer, 0.5 µL *Hind*III, 0.5 µL *Xho*I, 1000 ng of pAbAi, and topped off with sterile dH₂O. Digests were incubated at 37 °C for 3 hours and was DNA purified using the NucleoSpin® Gel and PCR Clean-Up Kit (Takara-Bio, Cat. # 740609.250) following the manufacturer's instructions. The linearized pAbAi was quantified using the ThermoFisher Nanodrop 1000. The PCR-amplified *FAR4* upstream regions were individually inserted into the linearized pAbAi vector using the In-Fusion HD Cloning Kit following manufacture's protocols. Two µL of the completed cloning reaction were immediately transformed into chemically competent *E. coli* (DH5α) ([2.2.4](#)). Colony PCR was used to confirm colonies containing successfully clones constructs ([2.2.10](#)) with primers listed in **Suppl. Table 3**. A single successful colony was amplified and vDNA (pBait-AbAi) was extracted from the culture ([2.2.7](#)). The constructs were verified via Sanger DNA sequencing (primers in **Suppl. Table 3**).

2.6.2- Construction of pGADT7-preyAD vectors

The Takara Bio In-Fusion Primer Design Tool was used to design primers (**Suppl. Table 3**) for the amplification of *MYB53*, *MYB92*, and *MYB93* full-length and N-terminal coding sequences, and subsequent insertion between the *EcoRI* and *BamHI* restriction sites of the pGADT7-AD vector (**Suppl. Figure 9**). Upstream regions were amplified from cDNA (preparation described above) prepared by Dr. Jhadeswar Murmu, using iProof™ High-Fidelity DNA Polymerase (Bio-Rad, Cat# 1725330), following the manufacturer's instructions. PCR products were column purified using the NucleoSpin® Gel and PCR Clean-Up Kit (Takara-Bio, Cat. # 740609.250) following the manufacturer's instructions. The PCR-amplified cDNA products were analyzed for quantity and purity using a ThermoFisher Nanodrop 1000. The pGADT7-AD vector was transformed into chemically competent DH5α *E. coli* cells, a single colony was amplified, and the pGADT7-AD vector was isolated ([2.2.4](#), [2.2.7](#)). *EcoRI* and *BamHI* restriction enzymes were used to digest pGADT7-AD vector in preparation for cloning. The 20 µL reaction contained: 2 µL 10X CutSmart Buffer, 0.5 µL *EcoRI*, 0.5 µL *BamHI*, 1000 ng of pGADT7-AD, and topped off with sterile dH₂O. Digests were incubated at 37 °C for 3 hours and was cleaned-up with the NucleoSpin® Gel and PCR Clean-Up Kit (Takara-Bio, Cat. # 740609.250) following the manufacturer's instructions. The linearized pGADT7-AD was quantified on the ThermoFisher Nanodrop 1000. The PCR-amplified cDNA regions were individually inserted into the linearized pGADT7-AD vector with the In-Fusion HD Cloning Kit following manufacture's protocols. Two µL of the completed cloning reaction were immediately transformed into chemically competent *E. coli* (DH5α) ([2.2.4](#)). Colony PCR was used to confirm colonies containing successfully cloned constructs ([2.2.10](#)) with primers from **Suppl. Table 3**. A single successful colony was grown, and vDNA (pGADT7-preyAD) was extracted and purified ([2.2.7](#)). The constructs were verified via Sanger DNA sequencing (primers in **Suppl. Table 3**).

2.6.3- Generating Y1H bait strains

To generate yeast bait strains, pBait-AbAi vectors generated above were integrated into the genome of the Y1HGold yeast strain (Genotype: MAT α , *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3,112*, *gal4 Δ* , *gal80 Δ* , *met-*, MEL1; Takara Bio). Each pBait-AbAi vector (**Suppl. Table 4**) was linearized with *Bst*BI for integration into the *ura3-52* locus, via recombination, of the Y1HGold genome. The 20 μ L linearization reaction contained: 2 μ L 10X CutSmart Buffer, 0.5 μ L *Bst*BI (20,000 units/mL), ~500 ng of pBait-AbAi vector, and topped off with sterile dH₂O. Digests were incubated at 65 °C for 3 hours and purified with the NucleoSpin® Gel and PCR Clean-Up Kit (Takara-Bio, Cat. # 740609.250) following the manufacturer's instructions. The linearized pBait-AbAi vectors were quantified on the ThermoFisher Nanodrop 1000. ~500 ng of each linearized pBait-AbAi vector was individually transformed into the Y1HGold yeast strain ([2.2.6](#)) and plated on SD (-URA) agar media. Colony PCR was done ([2.2.10](#)), with the primers in **Suppl.**

Table 3, to confirm the bait in the pBait-AbAi was correct. A second colony PCR was done with the Matchmaker Insert Check PCR Mix 1 (Cat. No. 630496, comes with own primers), as described by the manufacturer, to confirm the linearized pBait-AbAi vector was integrated into the yeast genome.

Each Y1HGold bait strain (**Suppl. Table 4**) was tested for the minimal concentration of Aureobasidin A required to completely inhibit its growth. A single large colony of each Y1HGold bait strain was re-suspended in 1 mL of sterile dH₂O, then diluted to an OD of 0.002. Dilution of 10 and 100 and were made, then 20 μ L of all three concentrations were spot plated on SD (-URA) agar media plates containing 100 ng/mL, 150 ng/mL, or 200 ng/mL of Aureobasidin A. The plates were left to grow for 3 days at 30 °C.

2.6.4- Transformation of pGADT7-preyAD vectors into the Y1HGold-pBaitAbAi strains

Each pGADT7-preyAD vector (**Suppl. Table 4**) was individually transformed into each Y1HGold bait strain ([2.2.6](#)) then then plated on SD (-Leu) agar media, and SD (-Leu) agar media containing the minimal inhibitory Aureobasidin A concentration for that Y1HGold bait strain.

2.7- Chemical analysis of suberin monomers in *wrky56* T-DNA loss-of-function mutants

2.7.1- Genotyping of T-DNA mutant lines

Seeds for three independent T-DNA mutant lines of *wrky56* were obtained from the Arabidopsis Biological Resource Center. A gene map for the following lines is shown in **Figure 13**: SAIL_737_DO1 (*wrky56-3*, T-DNA present in exon 1), SALK_201886C (*wrky56-1*, T-DNA present in the only intron), SAIL_1287_G04 (*wrky56-2*, T-DNA present in an exon 2). Left (LP) and right (RP) primers, used in combination with the common T-DNA left border primer (LbB1), were designed using the T-DNA Primer Design Tool (SIGnAL:SALK) and are listed in **Suppl. Table 3**. The *A. thaliana* (Col-0) seeds for each T-DNA mutant line were surface sterilized and grown ([2.2.3](#)). After ~2 weeks, leaf tissue was harvested from young seedling and gDNA was extracted ([2.2.8](#)). The genotype of individual plants for each T-DNA mutant line was determined via PCR ([2.2.10](#)) using primer combinations of LP + RP and LbB1 + RP (**Suppl. Table 4**) Individual plants that were determined to be homozygous for the T-DNA insertion in the gene of interest were left to grow, and the seeds were harvested. From these seeds', plants were grown for seed amplification, and re-genotyped to verify homozygous insertions prior to suberin analysis.

2.7.2- Root suberin monomer analysis

Sterilized *A. thaliana* (Col-0) WT and homozygous T-DNA mutant seeds (**Suppl. Table 1**) were stratified for 3 days in the dark at 4 °C, and then evenly spread on 0.5% agar plates containing ½ MS with vitamins (Phytotech Labs, Product ID: M404) and 1 % sucrose. One biological replicate (*n*) required six plates with ~40 seeds per plate, and each T-DNA mutant and WT *A. thaliana* (Col-0) seed line was analyzed using 4 biological replicates (*n*=4). Seeded plates were left under 24 hr light ($100\text{-}120\text{ }\mu\text{mol}\cdot\text{m}^2\cdot\text{s}^{-1}$, 21 °C) for 12 days.

The remaining experiments were all performed with HPLC-grade chloroform rinsed glassware. Roots for each biological replicate were harvested together, finely minced with a razor blade, and placed in pre-weighed glass tubes. The samples were incubated at 80 °C for 20 minutes in isopropanol (pre-heated to 80 °C) to inactivate lipases. The samples were cooled to room temperature and further homogenized with a hand-held homogenizer. Root samples were left to incubate, with rocking, in the isopropanol for 24 hours with a solution change after the first 8 hours. Roots were sequentially incubated in 2:1 (v/v) chloroform:methanol, 1:1 (v/v) chloroform:methanol, 1:2 (v/v) chloroform:methanol, and then 100% methanol to remove soluble lipids. The methanol was removed, and root samples were left to dry in a fume-hood for 3 days followed by a desiccator containing orange-indicating silica gel beads for at least 4 days. Root samples were considered fully dry once their weight stopped changing from day-to-day, and the final mass of root tissue was recorded.

For suberin depolymerization, 10-35 mg of solvent extracted, dry root tissue was used, which was transferred to new glass tubes. 20 µg of heptadecanoic acid and omega-pentalactone (which is linearized to omega hydroxy 15:0 fatty acid methyl ester during the depolymerization step) standards were added to each dry root sample along with 1.5 mL of depolymerization mix (60 % v/v methanol, 15 % v/v methyl acetate, 25 % v/v sodium methoxide). Suberin samples were vortexed vigorously and incubated at 60°C for 2 hours with vortexing every 30 minutes to cause depolymerization via base-catalyzed methanolysis. Suberin monomer samples were cooled to room temperature and then acidified to a pH between 4 and 5 with glacial acetic acid. Suberin monomers were extracted in 2.5 mL of dichloromethane, then washed three times with 2 mL of 0.5 M NaCl (keeping the organic phase each time), and anhydrous sodium sulphate added to remove any residual aqueous phase. The dichloromethane phase was dried under a gentle stream of nitrogen while heated to 37 °C. To acetylate the suberin monomers, they were re-suspended in 200 µL of 1:1 (v/v) pyridine: acetic anhydride,

vortexed, and incubated at 60 °C for 1 hour with vortexing every 15 minutes. Acetylated suberin monomers were dried under a gentle stream of nitrogen, while heated to 37 °C, and stored at -20 °C.

Acetylated suberin monomers were re-suspended in 200 µL of a 1:1 (v/v) toluene: heptane mixture and analyzed by gas chromatography on an Agilent Technologies 7820a GC system equipped with a flame ionization detector (FID). A 30 m by 0.25 mm HP-5 column, with 0.25 µm film thickness, was used and the carrier gas (He) had a 1.5 mL/minute flow rate. A 1 µL splitless injection was done at 300 °C and the detector had a temperature of 350 °C. The oven was programmed to hold for 3 minutes at 140 °C, raised to 310 °C at a rate of 10 °C/minute, and held for 17 minutes at 310 °C. Quantification of the suberin samples was based on the FID-generated peaks areas, relative to heptadecanoic acid methyl ester and acetylated 15-hydroxypentadecanoic fatty acid methyl ester internal standards. Composition of the suberin monomer samples was confirmed through GC-MS at the Carleton Mass Spectrometry Centre. Gas chromatography was performed exactly as described above but with MS detection performed using an Agilent Technologies 5977E MS detector. MS data was run through the database of the National Institute for Standards and Technology's Mass Spectrometry Data Centre.

3- Results

3.1- Transcriptional activation of suberin biosynthetic gene promoters

The first goal was to determine whether *Arabidopsis* MYB41, MYB53, MYB92, or MYB93 can transcriptionally activate the promoters (5' upstream regions from translation start site) of suberin biosynthetic genes. The full-length coding sequences of these transcription factors (and control transcription factors) were inserted into over-expression vectors (**Supp. Table 2, Supp. Fig. 6, 7, 8**) that were compatible with *Agrobacterium tumefaciens*, a bacterium capable of transferring T-DNA into a plant cell genome. The promoters of suberin biosynthetic genes *FAR4* (2300 bp), *GPAT5* (2164 bp), *ASFT* (1768 bp), *CYP86B1* (1523 bp), and *CYP86A1* (1788 bp), as well as the candidate *WRKY56* (1333 bp), were inserted into the *A. tumefaciens* compatible vector pGREENII 0800-LUC (**Supp. Fig. 2**) as transcriptional fusions with the *Firefly Luciferase* gene (Hellens *et al.*, 2005). In this system, when the T-DNAs harbouring the over-expressed transcription factors and promoter: reporter gene fusions are co-transferred into the same plant cell, we can test if the transcription factors transcriptionally activate the *Firefly Luciferase* reporter gene. The luminescence of the Firefly Luciferase protein is measured to quantify transcriptional activation (in the absence or presence of transcription factors). The pGREENII 0800-LUC vector also contains a *Renilla Luciferase* gene transcriptionally fused with a 35S promoter, and luminescence of the Renilla Luciferase protein is measured to normalize the amount of luminescence from the Firefly Luciferase protein between samples. Normalization was done by dividing the relative Firefly Luminescence by the relative Renilla Luminescence, and this is reported as Luciferase Activity.

The ability of *Arabidopsis* MYB39 (SUBERMAN) to activate the promoter of suberin biosynthesis genes using our transient *N. benthamiana* leaf dual-luciferase assay system was compared to similar work previously reported by Cohen *et al.* (2020). In my study, AtMYB39 increased luciferase activity of *GPAT5*, *ASFT*, *CYP86B1*, and *CYP86A1* promoters by 218-fold, 31-fold, 49-fold, and 68-fold, respectively, when

compared to the empty vector (EV) control (**Figure 4**). The increase in luciferase activity induced by AtMYB39 was statistically significant for each promoter, except for *CYP86A1*, when compared to the EV control. The work done by Cohen *et al.* (2020) was thus verified, establishing AtMYB39 as a positive control for our transient dual-luciferase promoter activation assays using AtMYB41, AtMYB53, AtMYB92, and AtMYB93.

Figure 4: Transient expression promoter activation assay, testing MYB39's ability to transcriptionally activate the promoter of *GPAT5*, *ASFT*, *CYP86A1*, *CYP86B1* and *WRKY56*. The y-axis displays the ratio of Firefly to Renilla Luciferase Activity. The transcription factors tested here are found in the legend (right side) and as bars indicated by their respective colours along the x-axis. The promoters are transcriptionally fused to Firefly Luciferase. The 35S promoter is transcriptionally fused to Renilla Luciferase and acts to normalize each individual replicate. Data in bars represent mean $\pm$ SE of 4 biological replicates, each from a different *N. benthamiana* plant. An asterisk indicates that the mean Luciferase Activity ratio (Firefly/Renilla) caused by a transcription factor is statistically different from the EV control. Statistical significance was found by first using the F-test to determine if variances between two groups were equal or unequal, followed by the two-tailed student t-test ($p < 0.05$).

All five *Arabidopsis* R2R3-MYB TFs (MYB39, MYB41, MYB53, MYB92, and MYB93) increased the Luciferase Activity (Firefly/Renilla ratio) of the *FAR4*, *GPAT5*, *CYP86A1*, *CYP86B1*, and *ASFT* promoters, when compared to the empty expression vector control (**Figure 5**). With the *FAR4* promoter, the Luciferase Activity was increased 9.0-fold, 6.1-fold, 5.1-fold, 3.6-fold, and 3.0-fold by AtMYB39, AtMYB41, AtMYB53, AtMYB92, and AtMYB93, respectively. With the *GPAT5* promoter, the Luciferase Activity was increased 40-fold, 13-fold, 17-fold, 10-fold, and 16-fold by MYB39, MYB41, MYB53, MYB92, and MYB93, respectively. With the *CYP86A1* promoter, the Luciferase Activity was increased 80-fold, 20-fold, 39-fold, 21-fold, and 114-fold by AtMYB39, AtMYB41, AtMYB53, AtMYB92, and AtMYB93, respectively. With the *CYP86B1* promoter, the Luciferase Activity was increased 104-fold, 78-fold, 88-fold, 50-fold, and 51-fold by AtMYB39, AtMYB41, AtMYB53, AtMYB92, and AtMYB93, respectively. With the *ASFT* promoter, the Luciferase Activity was increased 37-fold, 25-fold, 28-fold, 20-fold, and 47-fold by AtMYB39, AtMYB41, AtMYB53, AtMYB92, and AtMYB93, respectively. The mean Luciferase Activity caused by each R2R3-MYB TF, on each suberin biosynthesis gene promoter, was statistically compared to the corresponding empty vector (EV) controls to see if there were significant differences. If a R2R3-MYB TF caused a statistically significant change in Luciferase Activity, when compared to the EV control, an asterisk was placed above the bar in **Figure 5**. Most R2R3-MYB TF/'promoter' combinations caused a significant change in Luciferase Activity compared to the EV control. AtMYB39 did not significantly increase Luciferase Activity when tested on the *FAR4* and *GPAT5* promoters. The same occurred for AtMYB41 when tested on the *CYP86B1* promoter and AtMYB53 when tested on the *GPAT5* promoter. These four R2R3-MYB TF – suberin biosynthesis gene promoters combinations show activation but have a large standard error compared to the rest of the samples in **Figure 5**.

Figure 5: Transient expression promoter activation assay, testing MYB39, MYB41, MYB53, MYB92, and MYB93's ability to transcriptionally activate the promoter of *FAR4*, *GPAT5*, *ASFT*, *CYP86A1*, and *CYP86B1*. The y-axis displays the ratio of Firefly to Renilla Luciferase Activity. The transcription factors tested here are found in the legend (top right) and as bars indicated by their respective colours along the x-axis. The promoters are transcriptionally fused to Firefly Luciferase. The 35S promoter is transcriptionally fused to Renilla Luciferase and acts to normalize each individual replicate. Data in bars represent mean $\pm$ SE of 3-4 biological replicates, each from a different *N. benthamiana* plant. An asterisk indicates that the mean Luciferase Activity ratio (Firefly/Renilla) caused by a transcription factor is statistically different from the EV control. Statistical significance was found by first using the F-test to determine if variances between two groups were equal or unequal, followed by the two-tailed student t-test ($p < 0.05$).

Next, to assess specificity, the ability of non-suberin associated TFs to activate the *GPAT5* promoter (a gene unique to suberin biosynthesis) using the transient dual-luciferase assay system was tested. From *Arabidopsis*, MYB67 (subgroup at103;) and MYB45 (subgroup 16) were selected as they are not closely related to suberin-associated R2R3-MYB TFs, their over-expression does not result in an accumulation in suberin, and they are not expressed in the root endodermis (Kelemen *et al.*, 2015; Kosma *et al.*, 2014; Winter *et al.*, 2007). From *Poplar*, MYB134 and bHLH131 were chosen as they are a transcription factors that together activate many flavonoid promoters such as the *ANR1* gene, a gene required for

proanthocyanidin biosynthesis (James *et al.*, 2017). PtMYB134 and PtbHLH131 caused a 4.4-fold and 1.8-fold increase in Luciferase Activity on the PtANR1 promoter, respectively. In combination, PtMYB134 + PtbHLH131 caused a 185-fold increase in Luciferase Activity with the *PtANR1* promoter (**Figure 6A**), confirming what was determined by James *et al.* (2017). AtMYB45, AtMYB67, PtMYB134, PtbHLH131, and PtMYB134 + PtbHLH131 caused statistically non-significant changes in Luciferase activity of 0.92-fold, 1-fold, 1.6-fold, 1.9-fold, and 1.3-fold, respectively, on the *GPAT5* promoter when compared to the EV control (**Figure 6A**). AtMYB92 was used as a positive control, as it had the lowest activation strength on the *GPAT5* promoter (**Figure 5**) and induced a statistically significant 64-fold increase in Luciferase Activity on the *GPAT5* promoter (**Figure 6A**). The ability of AtMYB45 to induce a change in Luciferase Activity on the *CYP86A1*, *CPY86B1*, and *ASFT* promoters was also tested. No statistically significant changes to Luciferase Activity were found, and a 1.2-fold increase on the *CYP86B1* promoter was the highest recorded by MYB45 (data not shown).

Finally, the ability of suberin-associated R2R3-MYB TFs to activate the promoter of a non-suberin associated gene was tested to further define the specificity suberin-associated R2R3-MYB TFs have for suberin biosynthetic genes. The *ANR1* promoter, from *Populus trichocarpa* (*Pt*), which James *et al.* (2017) showed to be regulated by PtMYB134 + PtbHLH131 was used. Transcription factor combinations of PtMYB134, PtbHLH131, and PtMYB134 + PtbHLH131 acted as positive controls and demonstrated 3.2-fold, 1.7-fold, and 39-fold change in Luciferase Activity on the *PtANR1* promoter (**Figure 6B**), respectively. The change in Luciferase Activity caused by *PtMYB134* and *PtMYB134+PtbHLH131* were statistically significant. AtMYB39 and AtMYB93 caused statistically significant 5.8-fold and 5.8-fold increases in Luciferase Activity on the *PtANR1* promoter, compared to the EV control, respectively. AtMYB41, AtMYB53, and AtMYB92 caused statistically non-significant 1.6-fold, 4.3-fold, and 4.3-fold increases in Luciferase Activity, when compared to the EV control, respectively. When compared to the positive control combination of PtMYB134+PtbHLH131, the transcription factors PtMYB134, AtMYB39,

and AtMYB93 have a lesser ability to activate the luciferase reporter gene through the *PtANR1* promoter(Figure 6B).

Figure 6: Transient expression promoter activation assay testing A) the ability of non root suberin-associated TFs to transcriptionally activate *GPAT5* and B) the ability of suberin-associated R2R3-MYB TFs to transcriptionally activate the *PtANR1* promoter. The y-axis displays the ratio of Firefly to Renilla Luciferase Activity. The TFs tested here are found as bars indicated along the x-axis. The gene whose promoter upstream region is being tested is indicated under the x-axis. The promoters are transcriptionally fused to Firefly Luciferase. The 35S promoter is transcriptionally fused to Renilla Luciferase and acts to normalize each individual replicate. Data in bars represent mean $\pm$ SE of 4 biological replicates, each from a different *N. benthamiana* plant. An asterisk indicates that the mean Luciferase Activity ratio (Firefly/Renilla) caused by a transcription factor is statistically different from the EV control. Statistical significance was found by first using the F-test to determine if variances between two groups were equal or unequal, followed by the two-tailed student t-test ($p < 0.05$).

The five suberin-associated R2R3-MYB TFs were then tested for their ability to transcriptionally activate the *WRKY56* promoter. MYB39, MYB41, MYB53, MYB92, and MYB93 induced a 4-fold, 7-fold, 3.3-fold, 1.8-fold, and 4.4-fold change in Luciferase Activity with the *WRKY56* promoter transcriptionally fused to the Firefly Luciferase gene (**Figure 7**). The change in Luciferase Activity for MYB92 was not statistically significant when compared to the EV control.

Figure 7: Transient expression promoter activation assay, testing the root suberin-associated R2R3-MYB TFs ability to transcriptionally activate the promoter of *WRKY56*. The y-axis displays the ratio of Firefly to Renilla Luciferase Activity. The transcription factors tested here are found in the legend (right side) and as bars indicated by their respective colours along the x-axis. The *WRKY56* promoter is transcriptionally fused to Firefly Luciferase. The 35S promoter is transcriptionally fused to Renilla Luciferase and acts to normalize each individual replicate. Data in bars represent mean $\pm$ SE of 4 biological replicates, each from a different *N. benthamiana* plant. An asterisk indicates that the mean Luciferase Activity ratio (Firefly/Renilla) caused by a transcription factor is statistically different from the EV control. Statistical significance was found by first using the F-test to determine if variances between two groups were equal or unequal, followed by the two-tailed student t-test ($p < 0.05$).

3.2- Minimal *FAR4* promoter (upstream region) required for specific expression in root endodermis

The Arabidopsis *FAR4* gene is often used as a marker for sites of root suberin deposition (Domergue *et al.*, 2010b; Kosma *et al.*, 2014). Above, it was demonstrated that the 2300 bp upstream of the *FAR4* translation start site can be used by suberin-associated R2R3-MYB TFs to activate transcription of a downstream reporter gene (**Figure 5**). Next, I wanted to find potential binding sites for these R2R3-MYB TFs, if they do indeed bind directly to the *FAR4* promoter region. To narrow down the search, the minimal size of the 5' upstream region from the *FAR4* translation site (promoter) required for expression in the root endodermis was determined. *FAR4* promoters (*FAR4pro*) of -2300 bp, -2061 bp, -1594 bp, -1171 bp, -545 bp, and -254 bp were inserted into pKGWFS7 (**Figure 8**) to generate a 'promoter' deletion series. Typically the transcription start site, which is upstream of the translation start site, is designated '0' and nucleotide bases upstream are given a (-) designation and nucleotide bases downstream a (+) designation. For the most part, plant-based research has simplified the scope of the promoter to the region 5' of the translation start site, as done in this study. For that reason, the (-) in front of each number designates that the promoter is 5', or upstream, and the number represents the number of nucleotide bases from the translation start site. This means, that the transcription start site will have a (-) designation since it is upstream of the translation start site. With all nucleotide bases in the promoter being (-), the promoter is easier to define and search.

Figure 8: Schematic of the T-DNA region within the pKGWFS7 vector that the differently sized *FAR4* regions upstream of translation start site were inserted upstream of a GFP/GUS reporter gene fusion. The DNA between the left border (LB) and right border (RB) sites are transferred from *Agrobacterium* to plant genomic DNA.

The different sized *FAR4* promoters were transcriptionally fused with the β -Glucuronidase (*GUS*) gene, the reporter gene used here, within the pKGWFS7 vector. These recombinant plasmids were then transformed into *A. tumefaciens*, which in turn is used to incorporate the *FAR4pro: GUS* fusion within the T-DNA of the pKGWFS7 vector into the *Arabidopsis* genome via the floral dip method to generate stable transformant plant lines. Seeds recovered from ‘floral dipped’ plants were screened on Kanamycin media, and each seedling that survived represented an independent *FAR4pro: GUS* plant line (T1 generation). Multiple independent lines were analyzed for each promoter deletion (i.e. the same T-DNA harboring a *FAR4pro: GUS* fusion randomly incorporated into different positions of the *Arabidopsis* nuclear genome), and they are: -2300 bp (16), -2061 bp (18), -1594 bp (9), -1171 bp (14), -545 bp (16), and -245 bp (14) (# of independent T1 lines tested is indicated in brackets). The second generation (T2) of each independent *FAR4pro: GUS* line was stained for β -glucuronidase (*GUS*) activity. Entire 12-day old seedlings were imaged for *GUS* activity and one representative independent line for each *FAR4pro: GUS* construct in the deletion series is presented in **Figure 9**. Images for every independent line for each *FAR4pro: GUS* are found in **Supp. Table 5**. For the -2300 bp *FAR4pro*, *GUS* activity was found in the root endodermis, and at lateral root junctions, as was reported by Domergue *et al.* (2010), which matches the deposition of suberin at the same growth stage. Shortening *FAR4pro* to -2061 bp did not change the pattern or intensity of *GUS* activity, however a reduction to -1594 bp consistently decreased *GUS* activity seen in the root endodermis and the lateral root junctions. Further shortening of *FAR4pro* to -1171 bp did not cause an obvious change in *GUS* activity or pattern compared to the -1594 bp *FAR4pro*. Once down to -545 bp, the *FAR4pro* showed no expression in the root endodermis nor at lateral root junctions. No *GUS* activity was seen in the -245 bp *FAR4pro*. The regions of the *FAR4* promoter from -2061 bp to -1594 bp and -1171 bp to -545 bp were thus analyzed *in silico* for transcription factor *cis*-binding elements, with a focus on R2R3-MYB TF binding sites, as these regions of the *FAR4* promoter clearly influence root endodermis expression.

Figure 9: Staining pattern of the root tip, root maturation zone, and lateral root initiation of T_2 seeds containing the -2300 bp, -2061 bp, -1594 bp, -1171 bp, -545 bp, and -245 bp *FAR4* promoter fused to the β -glucuronidase (*GUS*) reporter gene. Seeds were grown for 12 days on $\frac{1}{2}$ MS agar with 50 μ g/mL Kanamycin and stained for GUS activity. Whole seedlings were incubated in the dark for 24 hr at 37 °C in a x-gluc buffer. Multiple independent lines were stained for each construct (Supp. Table 5), with only one representative line for each promoter deletion shown here.

3.3- Bioinformatic (in silico) analysis of the *FAR4* promoter for TF DNA-binding elements

R2R3-MYB, WRKY, and NAC transcription factors have previously been shown to influence suberin biosynthesis (Cohen *et al.*, 2020; Kosma *et al.*, 2014; Krishnamurthy *et al.*, 2020, 2021; Mahmood *et al.*, 2019; To *et al.*, 2020). With five R2R3-MYB TFs transcriptionally activating suberin biosynthesis genes (**Figure 5**) and *WRKY56* (Klein, 2019)(**Figure 7**), MYB and WRKY TF DNA-binding motifs were the focus of *cis*-element search of the suberin biosynthesis gene promoters. Kelemen *et al.* (2015) determined that *Arabidopsis* R2R3-MYB TFs have at least two different consensus DNA-binding motifs by analyzing all known R2R3-MYB TF interactions with DNA reported in the literature. The two consensus motifs are MYBcore (YNGTTR) and AC-rich (ACCWHH or ACCWAMY) (**Table 1**). Yeast 1-hybrid assays with synthesised DNA fragments showed that AtMYB53, AtMYB92, AtMYB93 preferred binding to AC-rich *cis*-elements, although they could bind to MYBcore *cis*-elements (Dubos *et al.*, 2014; Kelemen *et al.*, 2015). However, no in-depth analysis of how R2R3-MYB TF bind to specific motifs in suberin biosynthetic gene promoters have been done. Rather, MYB92 could bind and activate the promoter of a fatty acid

synthesis gene, *BCCP2*, and these actions required the presence of two adjacent AC-rich motifs (To *et al.*, 2020). WRKY33 and WRKY9 were found to bind to W-boxes with a sequence of TTGAC in the promoters of CYP86B1, CYP94B1, and CYP94B3 (Krishnamurthy *et al.*, 2020, 2021). Since some R2R3-MYB TFs are known to interact with bHLH TFs and a WD40-repeat protein to form a MBW complex, bHLH DNA-binding motifs were also searched. An exhaustive search using PlantPAN3.0 software (<http://plantpan.itps.ncku.edu.tw/promoter.php>), PLACE (<https://www.dna.affrc.go.jp/PLACE/?action=newplace>), and AGRIS (<https://agris-knowledgebase.org/AtcisDB/>) (Chow *et al.*, 2019; Higo *et al.*, 1999; Yilmaz *et al.*, 2011) was used to ensure no important binding site were missed (**Table 1**).

Table 1: Consensus DNA-binding motifs for R2R3-MYB, bHLH, NAC, and WRKY TFs

Class of Transcription Factor	Consensus DNA-Binding Motifs (Name)
R2R3-MYB (Kelemen <i>et al.</i> , 2015)	YNGTTR (MYB core) ACCWAMY (AC-rich) ACCWHH (AC-rich)
bHLH (Atchley & Fitch, 1997; Hartmann <i>et al.</i> , 2005)	CANNTG (E-box) CACNTG (G-box) NRCGTG (non E-box) domain CACGRG (N-box) GKKCKRWC (GCG-box)
NAC (Lindemose <i>et al.</i> , 2014; Zhong <i>et al.</i> , 2010)	CGTR (NAC core) CCCGCC (zinc-finger) TTVCTT (cluster 3) CTTN _{7/8} AAG (palindromic cluster 3) (T/A)NN(C/T) (T/C/G)TN ₇ A(A/C)GN(A/C/T) (A/T) →
WRKY (Elucidating the Evolutionary Conserved DNA-Binding Specificities of WRKY Transcription Factors by Molecular Dynamics and in Vitro Binding Assays, 2013; Kanofsky <i>et al.</i> , 2017; Van Verk <i>et al.</i> , 2008)	TGAC (core) or TTGACY GACTTT (WT-box core) TTTTCCAC (WK-box)

*Y = C/T, W = A/T, R = A/G, M = A/C, H = A/C/T, V = A/C/G, N = A/C/T/G

All of the promoters of genes transcriptionally activated by the suberin-associated R2R3-MYB TFs (**Figure 5**) contained MYB, WRKY, NAC, and bHLH transcription factor *cis*-elements (**Table 2**). Therefore, the enrichment of these motifs (**Table 1**) in the 3000 bp upstream from the translation start site ('promoter') of all suberin biosynthetic genes was done using the Analysis of Motif Enrichment (AME)

software, with default settings, from MEME Suite 5.3.3 (<https://meme-suite.org/meme/tools/ame>)

(**Supp. Table 6**). Compared to randomly generated control sequences, the R2R3-MYB TF AC-rich motif was enriched, but the MYBcore motif was not enriched (**Table 2**). No other consensus DNA-binding motifs from the bHLH, WRKY, and NAC TF families was enriched in the *FAR4* promoter analyzed.

Table 2: Amount of MYB, bHLH, NAC, and WRKY transcription factor *cis*-elements found in the promoter upstream region of suberin biosynthesis genes

Potential Binding TF	<i>Cis</i> -element (5'→3')	<i>ASFT</i> (3000 bp)	<i>GPAT 5</i> (2164)	<i>FAR 4</i> (2300 bp)	<i>CYP86 A1</i> (3000 bp)	<i>CYP86 B1</i> (3000 bp)
R2R3-MYB	YNGTTR	37	50	46	68	54
R2R3-MYB	ACCWHH	73	61	43	64	69
bHLH	CANNTG	36	34	18	57	40
WRKY	TGAC	31	39	18	27	24
WRKY	GGACTTTC	0	0	0	0	0
WRKY	TTTTCCAC	0	4	2	0	1
NAC	CGTR	41	59	29	47	27
NAC	CCCGCC	0	1	0	0	0
NAC	TTVCTT	20	17	7	11	21
CCAAT-box binding proteins	CCAAT	13	8	9	12	11
RNA-Polymerase II	TATAWAW	14	20	29	22	18

Since the AC-rich motifs are enriched in the suberin gene promoters and considering the results from the *FAR4* promoter deletion series, the -2061 bp to -1594 bp and -1171 bp to -545 bp regions of the *FAR4* promoter upstream region was broken up into multiple fragments based on the appearance of adjacent AC-rich motifs. There are six fragments of the *FAR4* promoter upstream region that became of most interest: -2037 bp to -1838 bp, -1825 bp to -1626 bp (**Figure 10**), -965 bp to -774 bp, -797 bp to -632 bp, -658 bp to -563 bp (**Figure 11**), and -551 bp to -401 bp (not shown). The last fragment from -551

to -401 bp, or bait #12, was outside of the determined range from the deletion series and was done out of curiosity as it still contained adjacent AC-rich MYB-DNA binding motifs.

Figure 10: To-scale map of the FAR4 promoter upstream region, -2061 bp to -1594 bp from the translation start site, identifying predicted DNA-binding motifs for MYB and WRKY TFs.

Figure 11: To-scale map of the FAR4 promoter upstream region, -1171 bp to -546 bp from the translation start site, identifying predicted DNA-binding motifs for MYB and WRKY TFs.

3.4- Yeast-one-hybrid assay to test for MYB TF binding to FAR4 upstream regions

The *FAR4* promoter fragments #3, #4, #9, #10, #11, and #12 (**Figure 10** and **11**) were inserted into the pAbAi vector, such that they were transcriptionally fused to the *Aureobasidin A* resistance gene (*AUR1-C*), to form pBait:AbAi vectors. The transcription factors (prey) being tested are fused with a yeast GAL4-activation domain (AD) in the pGADT7-AD vector. Any transcription factor capable of binding to the *FAR4* promoter fragments should activate *AUR1-C*, via the translationally fused GAL4-AD, and provide resistance to the antibiotic Aureobasidin A.

Each pBait:AbAi plasmid was linearized by the *Bst*BI restriction enzyme, which has a recognition site in the functional *URA* locus of the plasmid. The linearized pBait:AbAi vectors were transformed into Y1HGold yeast cells, where they recombine with the non-functional *ura3-52* locus in the Y1HGold genome to generate a functional *URA* locus. These transformed Y1HGold yeast cells were plated on media lacking uracil, and colonies were screened to confirm the pBait:AbAi vectors were successfully recombined into the Y1HGold genome to generate Y1HGold bait strains (**Figure 12**).

Figure 12: Colony PCR to confirm pBait-AbAi vector was integrated into the Y1HGold genome. PCRs were run on a 1 % agarose gel. A 1 Kbp DNA ladder (FroggaBio) was used to determine the size of each band. Band sizes were expected to be 1.4 – 1.5 Kbp.

To test the ability of an endogenous yeast transcription factor to bind to the *FAR4* promoter upstream region fragments and activate the Aureobasidin A resistance gene, each Y1HGold Bait strain was plated on SD (-Ura) media containing 100 ng/mL, 150 ng/mL, and 200 ng/mL of Aureobasidin A. None of the Y1HGold bait strains grew on SD (-Ura) containing any amount of Aureobasidin A, but they did grow on SD (-Ura) plates lacking Aureobasidin A as expected (**Figure 13**). The 100 ng/mL Aureobasidin A concentration was selected to test the ability of suberin-related R2R3 MYB TF to bind to the *FAR4* promoter upstream region fragments.

Figure 13: Y1HGold bait strains growing on SD (-Ura) media containing different concentrations of Aureobasidin A (AbA) in a dilution series. The yeast was plated as a dilution series (0.002, 0.0002 and 0.00002 OD_{600nm}). The *FAR4* promoter fragment numbers are indicated on the plates.

The full-length coding sequences of MYB53, MYB92, and MYB93 were inserted into pGADT7-AD (primers in **Supp. Table 3**) as translational fusions with the GAL4 Activation Domain. The full-length sequence of MYB92 and MYB53 were found to be toxic to the Y1H (yeast) cells. For this reason the N-terminal coding sequence for each R2R3-MYB TF was cloned into the pGADT7-AD vector because the N-terminal contains the DNA-binding domain and can still induce suberin production when overexpressed (Klein, 2019). If the transcription factors can bind to the *FAR4*pro fragment, the GAL4 AD will interact with the basal transcriptional machinery thereby activating transcription of *AUR1-C* and allowing the yeast to grow in media containing 100 ng/mL of Aureobasidin A (AbA).

Each transformation of prey vectors into the bait strains was plated onto two types of SD agar media: - Leucine and -Leucine with 100 ng/mL AbA (**Figure 14**). For the negative (H₂O) control, no yeast colonies grew on either type of media. For the EV (pGADT7-AD) control, colonies grew for each bait strain on the

-Leucine plates. For -Leucine + Aba plates the EV was found to grow well for bait #12 and slightly on bait #11, but no growth was found on the EV control for bait #3 and #9. Figure 12 is a representative figure from three trials for each type of transformation, and bait #4 and #10 are not shown below due to inconsistencies between the three trials. Bait #3 and #9 show no yeast growth on -Leu + Aba plates for MYB53 (N-term), MYB92 (N-term), and MYB93 (FL). Bait #11 showed some yeast growth at the highest cell density for the EV, but no yeast growth for MYB53 (N-term), MYB92 (N-term), and MYB93 (FL). On -Leucine + AbA media bait #12 with MYB53 (N-term), MYB92 (N-term), and MYB93 (FL) showed lots of yeast growth, but the EV did as well so binding cannot be confirmed. Bait #12 had a single colony growing at 100 ng/mL when determining the minimal inhibitory concentration of AbA for the bait strains, however it was not anticipated that this much autoactivation was occurring. Bait #12 will have to be tested at 150 ng/mL of AbA since 0 bait #12 colonies grew (**Figure 13**).

Figure 14: Y1H assay showing MYB53 (N-terminal), MYB92 (N-terminal), and MYB93 ability to bind to *FAR4* promoter fragments #3, #9, #11, and #12. pGADT7-AD empty vector was used as a negative control. The representative growth of yeast cells on SD/-Leu agar medium without and with 100 ng of Aureobasidin A at various relative densities is shown.

3.5- Chemical analysis of suberin in *wrky56* loss-of-function mutants

The names of the three *wrky56* T-DNA mutant line are: *SAIL_737_DO1* (*wrky56*-3: exon), *SALK_201886C* (*wrky56*-1: intron), *SAIL_1287_G04* (*wrky56*-2: exon). The location of the T-DNA insertion in the *WRKY56* gene for each mutant line is shown in **Figure 12**. The T_2 generation of each line was genotyped by PCR and only plants that were homozygous for the T-DNA insertion were used for suberin analysis. Sequencing still needs to be performed to confirm the precise locations of the T-DNA inserts, as well as transcript analysis to determine whether the T-DNA mutants are knockdowns or knockouts.

Figure 15: Location of the T-DNA in mutant *A. thaliana* (Col-0) seed lines used for the root suberin analysis. Each blue triangle represents a single T-DNA mutant. Grey areas are the UTR's, thick black lines are exons, and the thin black line is the intron.

The mean total aliphatic root suberin content for *wrky56-1*, *wrky56-2*, and *wrky56-3* T-DNA mutants were $127.79 \pm 8.97 \mu\text{mol/g}$, $123.37 \pm 3.17 \mu\text{mol/g}$, and $133.67 \pm 8.40 \mu\text{mol/g}$, respectively, none of which were significantly different from the $122.216 \pm 7.55 \mu\text{mol/g}$ of the wild-type plants (**Figure 16A; Supp. Table 7**). When total aliphatic suberin was broken down into classes of compounds (**Figure 16B; Supp. Table 8**), significant differences between the *wrky56-1* and *wrky56-3* T-DNA mutant lines and the wild-type plants were found in the ω -hydroxy fatty acids. The *wrky56-1* and *wrky56-3* mutants had $50 \pm 2.00 \mu\text{mol/g}$ and $48.39 \pm 2.08 \mu\text{mol/g}$, respectively, of ω -hydroxy fatty acids in the polyester, an increase from $40.66 \pm 2.32 \mu\text{mol/g}$ in the wild-type plants. This increase is due to an increase in 18:1 ω -hydroxy fatty acid (**Figure 17; Supp. Table 9**).

Figure 16: A) Total aliphatic suberin in roots of 12-day old *Arabidopsis* seedlings comparing wild-type to three different *wrky56* T-DNA insertion alleles. No periderm expected in this young age of plants, and therefore suberin is from root endodermis B) Suberin monomer levels sorted into compound class, comparing wild-type to the three *wrky56* T-DNA insertion alleles. Bars represent μmol/g of DW (dry-weight) ± SE and asterisks are placed above bars that are statistically different (F-Test and two-tailed t-test) from the WT (wild-type) control.

Figure 17: Amount of individual ω -hydroxy fatty acids found in root suberin of wild-type and three wrky56 T-DNA insertion alleles of *Arabidopsis*. Bars represent $\mu\text{mol/g}$ of DW (dry-weight) $\pm$ SE and asterisks are placed above the bars that are statistically different (F-test and two-tailed t-Test) from the WT (wild-type) control.

4- Discussion

4.1- Opening Statement

The root endodermis of *Arabidopsis* contains aliphatic suberin and lignified Casparian strips in its cell walls. These are hydrophobic barriers that direct the movement of water and nutrients through the symplastic pathway and into the xylem (Barberon *et al.*, 2016; Barbosa *et al.*, 2019). SHORT-ROOT (SHR) is a GRAS-family transcription factor that is necessary for root endodermis identity throughout development. Expressed in the central root stele, SHR travels symplastically to the endodermis where it activates MYB36, although not directly, as part of stage I of root endodermis differentiation (Li *et al.*, 2018). MYB36, an R2R3-MYB transcription factor, directly induces the expression of *CASP1*, *PER64*, and *ESB1*, which encode enzymes responsible for the polymerization of Casparian strips (Kamiya *et al.*, 2015). A second GRAS-family transcription factor SCARECROW (SCR), also regulated by SHR, regulates genes encoding receptor-like kinases (SNG1/SNG3) in the plasma membrane that trigger Casparian strip formation. The SCR pathway is mediated by stele-derived peptides CIF1/CIF2 (independent of SHR), that migrate to the endodermis through the apoplast and interact with SNG1/SNG3 to direct Casparian strip formation in a ring around the anticlinal cell wall of endodermal cells, thereby sealing the entire apoplast between adjacent endodermal cells (Kamiya *et al.*, 2015; Li *et al.*, 2018; Naseer *et al.*, 2012). Once a Casparian strip is formed these peptides can no longer leave the stele, ensuring Casparian strip formation is specific to the endodermis (Li *et al.*, 2018).

While stage I of root endodermis differentiation involves blocking the apoplast with Casparian Strips, stage II involves the deposition of aliphatic suberin all around cells of the root endodermis to block the transmembrane movement of water and nutrients (Naseer *et al.*, 2012). Interestingly, the overexpression of *iSHR* and *iMYB36* eventually lead to the production of functional suberin in the root endodermis (C. Wang *et al.*, 2020). Transcript analysis of *iSHR* and *iMYB36* revealed that most suberin

biosynthesis genes were upregulated along with suberin regulators MYB9, MY39, MYB41, MYB93, and MYB107, and MYB39. Mutant and overexpression line transcript analysis also provide evidence MYB39 functions downstream of SHR/MYB36 (Cohen *et al.*, 2020; C. Wang *et al.*, 2020). The regulation of suberin deposition being a downstream target of the SHR/MYB36 regulatory pathway makes sense for the timing and development of the root endodermis, but a lot is still unknown. With suberin having a complex biosynthetic pathway, transport, and cell wall assembly, plus having roles in lateral root development, abiotic and biotic stress responses, suberin production is likely controlled by an intricate regulatory network (Geldner, 2013; Vishwanath *et al.*, 2015). While each enzyme that is involved in suberin biosynthesis can be regulated at various levels (transcriptional, post-transcriptional, translational, and post-translational), most research to date has focused on their transcriptional regulation. While MYB36 directly regulates Casparian Strip biosynthetic enzymes (Kamiya *et al.*, 2015), it appears that other R2R3-MYB TF have a similar role in suberin biosynthesis. R2R3-MYB TFs of subgroup 10 (MYB9, MYB39, MYB107), subgroup 11 (MYB41, MYB74, MYB102), and subgroup 24 (MYB53, MYB92, MYB93) have all been implicated in positively regulating suberin biosynthesis in *Arabidopsis*. However, little work has been done to determine whether these transcription factors are direct or indirect trans-activators of suberin biosynthesis genes or how they may work with other transcriptional regulators (Cohen *et al.*, 2020; Kosma *et al.*, 2014; Lashbrooke *et al.*, 2016; Murmu *et al.*, manuscript in preparation; To *et al.*, 2020). My study was focused on these mechanistic gaps in our understanding of the regulated deposition of suberin.

4.2- Suberin-associated MYB transcription factors trans-activate the promoter upstream regions of suberin biosynthetic genes

Transcription factors that promote suberin biosynthesis in seeds coats (MYB9 and MYB107), the root endodermis under abiotic stress (MYB41), and the root endodermis throughout development (MYB39) have been reported, covering most of spatial-temporal occurrences of suberin in *Arabidopsis* (Cohen *et*

al., 2020; Gou *et al.*, 2017; Kosma *et al.*, 2014; Lashbrooke *et al.*, 2016). Loss of function mutants for MYB107 and MYB9 were used to demonstrate a decrease in seed coat suberin and associated suberin gene mRNA transcripts, as well as increased sensitivity of the mutants to osmotic stress, which is in line with the role of seed coat suberin (Gou *et al.*, 2017; Lashbrooke *et al.*, 2016). Ectopic over-expression of MYB41 in leaves was used to show its ability to induce the expression of suberin gene mRNA transcripts, individual suberin monomers, and suberin lamellae, but *MYB41* is only expressed in the root endodermis in response to abiotic (salt) stress (Kosma *et al.*, 2014). Cohen *et al.* (2020) used a loss-of-function mutant and over-expression lines to demonstrate that MYB39 induces suberin biosynthesis throughout root development, and they also demonstrated the trans-activation capabilities of MYB39 on suberin gene promoters. Specifically, MYB39 activated gene expression when a reporter gene (luciferase) was fused with the promoters (region 5' of translation start site) of suberin biosynthetic genes *FAR4*, *FAR1*, *GPAT5*, *ASFT*, and *CYP86B1*, as well as the suberin transport gene *ABCG6* (Cohen *et al.*, 2020).

Similar to MYB39, Arabidopsis MYB53, MYB92 and MYB93 have also been implicated in regulating suberin biosynthesis in root endodermis through loss-of-function mutants, over-expression lines, and promoter: reporter gene expression experiments (Hu, 2018; Klein, 2019; Murmu *et al.*, manuscript in preparation, Rowland Lab). My study here reproduced the finding by Cohen *et al.* (2020) that MYB39 can transactivate suberin gene promoters (**Figure 4**). I also demonstrated that MYB41, MYB53, MYB92, and MYB93 also transcriptionally activate suberin biosynthesis gene promoters (specifically, *FAR4*, *GPAT5*, *CYP86A1*, *CYP86B1*, and *ASFT*) (**Figure 5**). The next step would be to test the ability of each root suberin-associated MYB TF to transcriptionally activate genes encoding suberin transporters (*ABCG2*, *ABCG6*, *ABCG20*) and suberin polymerization enzymes (*GELP22*, *GELP38*, *GELP49*, *GELP51*, *GELP96*) genes, and even fatty acid synthesis genes, to see if each TF regulates every step or just parts suberin deposition. Also, MYB45 and MYB67 could not transcriptionally activate *GPAT5* (**Figure 6A**), which is

consistent with the findings of Kosma *et al.* (2014) who over-expressed MYB45 and MYB67 and observed no suberin monomer accumulation. R2R3-MYB and bHLH TF from *Poplar* also could not trans-activate *GPAT5* (**Figure 6B**), however, transcription factors that transcriptionally regulate similar surface lipid barriers (e.g. cuticle) or the closely associated lignified polymer that makes up Casparian Strips should be used to further evaluate the specificities of MYB-type TF that can trans-activate suberin biosynthetic genes. Further characterization of the expression patterns of MYB39, MYB41, MYB53, MYB92, and MYB93 during development and in response to all types of stress, and if they match the expression of suberin biosynthesis genes, needs to be analyzed to carve-out any unique roles for each TF. It should be noted that statistical significance throughout this study was determined using the F-test followed by a two-tailed student t-test, as done by Cohen *et al.* (2020), which can be prone to false-positives.

4.3- No evidence of Arabidopsis MYB53, MYB92, or MYB93 binding to the FAR4 promoter using yeast 1-hybrid analysis

Although the root suberin-associated MYB TFs are transcription activators of suberin biosynthetic gene promoters, this does not necessarily mean they have a direct and physical interaction with the cognate promoters. There may be a transcriptional cascade where one or any combination of root suberin-associated MYB TFs activate each other, or another transcription factor, which then binds to promoter to activate the transcription of the suberin biosynthesis genes. Chromatin immunoprecipitation-qPCR and Y1H revealed that MYB107 directly binds to the promoter of suberin biosynthesis genes (*GPAT5*, *FAR1*, and *FACT*) (Gou *et al.*, 2017). Y1H experiments were done to test whether MYB39 could directly bind to promoter upstream regions of 10 suberin biosynthetic genes and it was only found to bind to the *FAR5* promoter upstream region (C. Wang *et al.*, 2020). Here, I attempted to determine if MYB53, MYB92, or MYB93 could directly bind to the promoter upstream region of suberin biosynthesis gene *FAR4*, through a Y1H, which I previously demonstrated was transcriptionally activated by these three

R2R3-MYB transcription factors (**Figure 5**). Y1H was used instead of ChIP-qPCR due to an inability to epitope tag the root suberin-associated R2R3-MYB TFs. Epitope tags on the N-terminus or C-terminus either caused a loss of activity or the epitope tags were removed by proteolysis in the cell (Rowland, personal communication). Also, antibodies specific to these transcription factors are not available.

The *FAR4* promoter deletion series experiment indicated that the -545 bp to -1171 bp and -1594 to -2061 bp regions (**Figure 9**) contain important transcription factor binding sites for expression of *FAR4* in root endodermis, which matches the sites of aliphatic suberin deposition in this tissue. These regions also contain numerous potential AC-rich MYB DNA-binding motifs (**Figure 10 and 11**), however only the -1594 to -2061 bp region contained occurrences where two AC-rich MYB-DNA binding motifs were adjacent to each other as required by MYB92 to bind to the *BCCP2* promoter (To *et al.*, 2020). The bait fragments generated covered a majority of these two regions in the *FAR4* promoter, however three (bait #3, #9, and #11) were not bound by MYB53, MYB92, and MYB93 using the Y1H assay, while more conclusive evidence is still needed for baits #4 and #10. As for bait #12, no conclusion can be made due to autoactivation by the EV that occurred. While it is possible the root suberin-associated R2R3-MYB TFs may not bind the *FAR4* promoter, other factors may be influencing this negative result. *Arabidopsis* MYB53, MYB92, and MYB93 having very high sequence similarity in their R2R3 DNA-binding domains and likely bind to DNA in a similar fashion. Genome-wide cistrome and epicistrome analysis of *Arabidopsis* TFs by O'Malley *et al* (2016) included the target genes of MYB93. Through DNA-affinity purification (DAP) of native gDNA, MYB93 was bound to the *FAR4* promoter but was not bound to the *FAR4* promoter when the native gDNA was PCR amplified before the DAP (amp-DAP). Native gDNA contains chemical modifications, such as methyl-cytosine, which are removed during PCR amplification. Comparing the binding of MYB93 in DAP vs. amp-DAP states indicates that an epigenetic chemical modification is needed for MYB93 to bind to the *FAR4* promoter (**Supp. Table 7**). Interestingly, MYB93 was bound to the *BCCP2* promoter region in the amp-DAP state but not in the DAP state (O'Malley *et al.*,

2016)(**Suppl. Table 7**). The methylation of cytosine is a major chemical modification made to DNA and AC-rich motifs being the preference for MYB53, MYB92, and MYB93 meaning very important cytosine residues may not be modified (i.e. methylated) thereby preventing binding to occur in the Y1H assays. The full-length coding sequence of MYB92 and MYB53 appeared to be toxic when expressed in the yeast bait strains, although this was not the case for MYB93. For this reason, only the N-terminal halves of MYB92 and MYB53, which contains the predicted R2R3 DNA-binding domain, were used for the Y1H assays and it is possible that the loss of the C-terminal portion affects the ability of the transcription factors to bind DNA despite lacking any obvious DNA binding domains (Millard *et al.*, 2019).

There are other reasons that no binding was observed in the Y1H assay. First, the 100 ng/mL of AbA used as the minimal concentration to eliminate auto-activation of the *AUR1-C* gene (Aba resistance gene) by endogenous yeast transcription factors may be high enough to prevent any activation caused by binding of any of the three R2R3-MYB TFs. Testing a concentration range from 50 – 100 ng/mL may reveal binding of these transcription factors, compared to the empty vector negative control. Second, the *FAR4* promoter fragments tested in the Y1H contain all the predicted AC-rich R2R3-MYB TF DNA binding sites from the manual analysis performed, but there could still be DNA-binding sites for MYB TFs not yet reported. Other regions of the *FAR4* promoter may thus need to be tested. Third, the R2R3-MYB TFs may need one or more partner transcription factors to bind the *FAR4* promoter. The binding of MYB92 to the *BCCP2* promoter suggested dimerization (although not confirmed) due to the adjacent AC-rich sites (To *et al.*, 2020). However, *FAR4* is not a fatty acid synthesis gene like *BCCP2* and it is possible they are regulated differently such that a hetero-dimer and not a homo-dimer is required for binding to the *FAR4* promoter. R2R3-MYB TFs have been shown to interact with bHLH and WRKY TFs to regulate transcription of some genes (Amato *et al.*, 2019; Vannozzi *et al.*, 2018), and recently it was shown that MYB92 and MYB93 interact with bHLH111 through a yeast 2-hybrid assay (Tapp, 2020 – Rowland Lab).

Outside of the highly conserved N-terminal R2 and R3 DNA-binding domains (DBD) in R2R3-MYB TFs, little is known about the function of the rest of the protein which is highly variable. The motif ([DE]Lx2[RK]x3Lx6Lx3R within the R3 DBD is required for interactions with R/B-like bHLH TFs (Zhao *et al.*, 2013). However, a second study found that subgroup 12 R2R3-MYB TFs and closely related MYB95 and MYB47 contain a [L/F]LN[K/R][V/L]A motif in the C-terminal non-MYB (or non-R2R3 DBD region) required for interactions with the bHLH TFs MYC2, MYC3, and MYC4. This has led to the idea that MYB-bHLH interactions have evolved independently of each other, based on the specific needs of a sub-set of similar functioning R2R3-MYB TFs, making it difficult to predict MYB-bHLH interactions (Millard *et al.*, 2019). As previously mentioned, R2R3-MYB TFs can also work with WRKY-family TFs to regulate gene expression. In soybeans, it was found through yeast 2-hybrid analysis that MYB174 and WRKY27 specifically interact with each other. Together, they bind and suppress the expression of *NAC029*, which in-turn activates *SPOD1* to reduce intracellular ROS levels and improving stress tolerance in soybean (Wang *et al.*, 2015). However, nothing is yet reported on how or if MYB174 and WRKY 27 physically interact. Therefore, the removal of the C-terminal half of MYB53 and MYB92 may affect their ability to homo or hetero-dimerize, thus preventing binding to the *FAR4* promoter.

4.4- Arabidopsis WRKY56 may have a role in regulating suberin deposition in response to abiotic stress

WRKY56 is thought to be a down-stream target of suberin-associated R2R3-MYB TFs (Cohen *et al.*, 2020; Klein, 2019; Lashbrooke *et al.*, 2016). In this study, three independent *wrky56* T-DNA mutants were obtained to determine if *WRKY56* plays a role in regulating root suberin biosynthesis. None of the *wrky56* T-DNA mutants had a significant difference in overall root suberin content when compared to WT (**Figure 16A**), but some individual compounds were altered. The *wrky56-2* mutants had a small decrease in 16:0 and 18:0 fatty acids, but there were no changes to compounds more unique to suberin. In this allele, the T-DNA is outside the DNA-binding domain or any known functional domain of *WRKY56*,

so it may not be a knock-out allele. The *wrky56-3* mutant had a specific increase in 18:1 ω -hydroxy fatty acids (**Figure 17**), which was accompanied by a decrease in 16:0 and 18:1 fatty acid (**Suppl. Table 8**). The *wrky56-1* mutant also had an increase in 18:1 ω -hydroxy fatty acids, but no compounds had a significant decrease. The increases in 18:1 ω -hydroxy fatty acids indicated WRKY56 may be a negative regulator of suberin biosynthesis, and may specifically act on *CYP86A1* (Höfer *et al.*, 2008). The difference between *wrky56-1* and *wrky56-3* and the *wrky9* and *wrky33* mutants is that *wrky56* only increased 18:1 ω -hydroxy fatty acid, while *wrky9* and *wrky33* decreased 16:0 ω -hydroxy fatty acids and altered α , ω -dicarboxylic acids and fatty alcohol content (Krishnamurthy *et al.*, 2020, Krishnamurthy *et al.*, 2021). In the *wrky56-1* and *wrky56-3* alleles the T-DNA is in the intron and exon 1, respectively. The *wrky56-1* allele may be affecting intron splicing and the *wrky56-3* allele is likely interfering the DNA-binding domain of WRKY56. However, it has yet to be confirmed whether the *wrky56* mutants are knockdown or knockouts and so a complete picture of WRKY56's impact on suberin under non-stress conditions cannot be made. Another way to screen for WRKY56's potential role in regulating suberin deposition would be to overexpress MYB53 (or any other root suberin associated R2R3-MYB TF) with and without WRKY56, in *N. benthamiana* leaves, and see if suberin deposition is different between the two samples.

All of the suberin-associated R2R3-MYB TF tested here (MYB39, MYB41, MYB53, MYB92 and MYB93) could transcriptionally activate the *WRKY56* promoter, although the amount of Luciferase Activity was significantly lower than what was observed with the suberin biosynthesis gene promoters (**Figure 5**). Since MYB41, which is abiotic stress induced, transcriptionally activates the *WRKY56* promoter, it should be noted that the potential involvement of MYB53, MYB92, and MYB93 in abiotic stress responses are not known. Expression data from ePlant (<https://bar.utoronto.ca/eplant/>) shows that *WRKY56* is highly upregulated in the roots in response to various abiotic stresses (i.e. cold, drought, salt, osmotic). Although direct binding studies still need to confirm if the suberin-associated R2R3-MYB TFs are direct regulators of *WRKY56*, it is likely that *WRKY56* is down-stream in their regulatory pathway. The ability of

WRKY56 to transcriptionally activate the promoter of suberin biosynthesis genes, specifically CYP86A1 which is responsible for 18:1 ω -hydroxy fatty acid production, should be done to complement the suberin analysis in *wrky56* roots. Also, performing the chemical and microscopic analysis of root suberin after applying abiotic stress to *wrky56* mutant versus control wild type, may give a more clear picture on the role of WRKY56 in regulating suberin biosynthesis.

4.5- Conclusions

I determined that *Arabidopsis* MYB53, MYB92, and MYB93, as well as MYB41, transcriptionally activate the promoter upstream regions of suberin biosynthesis genes. I also mapped two 500-600bp regions of the *Arabidopsis* *FAR4* promoter that are important for directing specific gene expression in root endodermis. Although I found that MYB TF AC-rich motifs are enriched in the promoter upstream regions of suberin biosynthesis genes, including in the two regions of the *FAR4* promoter required for root endodermis expression, there was no evidence that MYB53, MYB92, or MYB93 binds directly to the *FAR4* promoter using yeast 1-hybrid assays. However, more research needs to be done with the yeast 1-hybrid assay before any firm conclusions can be made, and ideally an alternate approach should be taken. Lastly, I found that *Arabidopsis* *WRKY56* promoter to be transcriptionally activated by these same root suberin-associated R2R3-MYB transcription factors. Chemical root suberin analysis of *wrky56* mutants indicated that WRKY56 is a negative regulator that specifically impacts ω -hydroxy fatty acid production. However, the overall impact of WRKY56 on the suberin polymer under normal developmental conditions appears to be minimal, and further analysis should be done with a focus on the potential role of WRKY56 in regulating suberin deposition under abiotic stress conditions and through overexpression experiments.

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6- Supplemental Material

Supplemental Table 1: Seed lines

Species	Genotype	# of independent lines
<i>Nicotiana benthamiana</i>	Wild type	N/A
<i>Arabidopsis thaliana</i> (Col-0)	Wild type	N/A
<i>Arabidopsis thaliana</i> (Col-0)	<i>FAR4</i> promoter(-2300bp): GUS	16
<i>Arabidopsis thaliana</i> (Col-0)	<i>FAR4</i> promoter(-2061bp): GUS	18
<i>Arabidopsis thaliana</i> (Col-0)	<i>FAR4</i> promoter(-1594bp): GUS	9
<i>Arabidopsis thaliana</i> (Col-0)	<i>FAR4</i> promoter(-1171bp): GUS	14
<i>Arabidopsis thaliana</i> (Col-0)	<i>FAR4</i> promoter(-545bp): GUS	16
<i>Arabidopsis thaliana</i> (Col-0)	<i>FAR4</i> promoter(-245bp): GUS	14
<i>Arabidopsis thaliana</i> (Col-0)	<i>wrky56-1</i> T-DNA mutant (SALK_201886C)	N/A
<i>Arabidopsis thaliana</i> (Col-0)	<i>wrky56-2</i> T-DNA mutant (SAIL_1287_G04)	N/A
<i>Arabidopsis thaliana</i> (Col-0)	<i>wrky56-3</i> T-DNA mutant (SAIL_737_D01)	N/A

Supplemental Table 2: DNA vectors, host strains, and required antibiotics

Vector	Host Species	Selection (µg/mL)
pMDC32	<i>E. coli</i> (DH5α)	Kanamycin (50)
	<i>A. tumefaciens</i> (GV3101: pMP90)	Rifampicin (100), Gentamycin (40), Kanamycin (50)
pK7WG2D	<i>E. coli</i> (DH5α)	Spectinomycin (80)
	<i>A. tumefaciens</i> (GV3101: pMP90)	Rifampicin (100), Gentamycin (40), Spectinomycin (100)
pKGWFS7	<i>E. coli</i> (DH5α)	Spectinomycin (80)
	<i>A. tumefaciens</i> (GV3101: pMP90)	Rifampicin (100), Gentamycin (40), Spectinomycin (100)
	<i>A. thaliana</i> (Col-0)	Kanamycin (50)
pGREENII 0800-LUC	<i>E. coli</i> (DH5α)	Kanamycin (50)
pSOUP	<i>E. coli</i> (DH5α)	Tetracycline (5)
pGREENII 0800-LUC + pSOUP	<i>A. tumefaciens</i> (GV3101: pMP90)	Rifampicin (100), Gentamycin (40), Kanamycin (50), Tetracycline (2.5)
pBIN19-p19	<i>A. tumefaciens</i> (C58C1+pCH32)	Kanamycin (50), Tetracycline (2.5)
pGADT7-AD	<i>E. coli</i> (DH5α)	Ampicillin (100)
	<i>S. cerevisiae</i> (Y1HGold)	-Leucine
pAbAi	<i>E. coli</i> (DH5α)	Ampicillin (100)
	<i>S. cerevisiae</i> (Y1HGold)	- Uracil

Supplemental Table 3: Primer list

Stock #	Primer Name	Primer Sequence (5' --> 3')	Use
210	FAR4Prom-Forward-attaB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTG GAACCTATGTCCGAACCTCC	FAR4 promoter Gateway Cloning
211	FAR4Prom-Reverse-attaB2	GGGGACCACTTTGTACAAGAAAGCTGGGTG AAGAACTTATATCTATCC	FAR4 promoter Gateway Cloning
213	FAR4_Foward_1	GGGGACAAGTTTGTACAAAAAAGCAGGCTCT CCGAGATTTTCAGAGCATG	FAR4 promoter Gateway Cloning
214	FAR4_Foward_2	GGGGACAAGTTTGTACAAAAAAGCAGGCTAT TAGAACGCCAACCTTCCA	FAR4 promoter Gateway Cloning
215	FAR4_Foward_3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTT GTCAAACATCTGCATTGT	FAR4 promoter Gateway Cloning
216	FAR4_Foward_4	GGGGACAAGTTTGTACAAAAAAGCAGGCTG ACTTGTACTTCTAAAACCTC	FAR4 promoter Gateway Cloning
217	FAR4_Foward_5	GGGGACAAGTTTGTACAAAAAAGCAGGCTA GCAGTTCTAACCCCGACAT	FAR4 promoter Gateway Cloning
237	pKGWFS7-SeqFor	ATCCTCTAGAGTCGACCTGCA	Colony PCR/Sequencing for pKGWFS7
238	pKGWFS7-SeqRev	GAAGTTGTGGCCGTTTACGTC	Colony PCR/Sequencing for pKGWFS7
1406	AtFAR4 promoter LUC FW (HindIII)	CGGTATCGATAAGCTTGGAACCTATGTCCGA AC TC	Infusion cloning into pGREENII 0800-LUC
1407	AtFAR4 promoter LUC RV (BamHI)	TAGAACTAGTGGATCCTGAAGAACTTATAT CTATCCAA TT	Infusion cloning into pGREENII 0800-LUC
1410	AtGPAT5 promoter LUC FW (HindIII)	CGGTATCGATAAGCTTTGATCGCAAACGTCA ATGGT	Infusion cloning into pGREENII 0800-LUC

1411	AtGPAT5 promoter LUC RV (BamHI)	TAGAACTAGTGGATCCTTCTTTTGT TTTTGT CGAATA TT	Infusion cloning into pGREENII 0800-LUC
1609		TTTCTTAAATCAATAAGCTTTGATGTACTGA	Infusion cloning into pGREENII 0800-LUC
1610	BamHI-ASFT-P-R	TTTGATCCAAATGGAGAAAACAGCAC	Infusion cloning into pGREENII 0800-LUC
1611	HindIII-RALPH- P-F	AGTACTTTACTTACTCGGTAGCACACC	Infusion cloning into pGREENII 0800-LUC
1612	BamHI-RALPH- P-R	TGTGACAAAGAGAAGAGAGAGCGATG	Infusion cloning into pGREENII 0800-LUC
1613	HindIII-HORST- P-F	TGCCAAAACCATGCACGATCTTGT	Infusion cloning into pGREENII 0800-LUC
1614	BamHI-HORST- P-R	TATCCCGGTTTAGGCTTTTGTCTTTGT	Infusion cloning into pGREENII 0800-LUC
1615	HindIII-WRKY56- P-F	TTCGAGCGGTCAAAGAGATTCTTAAC	Infusion cloning into pGREENII 0800-LUC
1616	BamHI- WRKY56-P-R	TGATTATGAAGCTTTCACATCTTGCTAG	Infusion cloning into pGREENII 0800-LUC
1528	pGREENII0800L UC_SeqFW	CCAGTCACGACGTTGTAA	Colony PCR/sequencing for pGREENII 0800-LUC
1529	pGREENII0800L UC_SeqRV	CCTCGATATGTGCATCTG	Sequencing for pGREENII 0800- LUC
1525	GPAT5pRV	CATCCTCGGCACCATCC	Colony PCR for pGREENII 0800- LUC

1526	FAR4pRV	TGCATCGGACATATCAAGCC	Colony PCR for pGREENII 0800-LUC
1617	ASFT-Pro-R1	CTTCAAAATTCAAATCCACACTG	Colony PCR for pGREENII 0800-LUC
1618	RALPH-Pro-R1	TGAGGATTGTCCTAAGTTTGGAA	Colony PCR for pGREENII 0800-LUC
1619	HORST-Pro-R1	TCAGATCACCCAATCCTAAAAAAT	Colony PCR for pGREENII 0800-LUC
1620	WRKY56-Pro-R1	GTCGGTTTGAGAACTAGCTAAAATT	Colony PCR for pGREENII 0800-LUC
1625	MYB39-CDS-F		Cloning into pDONR221
1626	MYB39-CDS-R		Cloning into pDONR221
578	35S:Promo-F-pK7WG2D	GAGGACTCCGGTATTTTACAAC	Colony PCR/Sequencing for pK7WG2D
579	35S:Term-R-pK7WG2D	CCCTAATTCCTTATCTGGGAAC	Colony PCR/Sequencing for pK7WG2D
1627	FAR4proY1H#3 FW	ATGAATTGAAAAGCTTGATAGGGCAGCGGTGTATGG	Cloning into pAbAi
1628	FAR4proY1H#3 RV	GAGCACATGCCTCGAGCAACTGTAAAAAGAGCCTTAGGG	Colony PCR /Cloning into pAbAi
1629	FAR4proY1H#4 FW	ATGAATTGAAAAGCTTTCAGTTCGCTATTGAA GTATACATG	Cloning into pAbAi
1630	FAR4proY1H#4 RV	GAGCACATGCCTCGAGGCAAAGTTACACAGATGGTAACAG	Colony PCR/Cloning into pAbAi
1631	FAR4proY1H#9 FW	ATGAATTGAAAAGCTTGCTATTATTTAAGTAACACTAACAA	Cloning into pAbAi
1632	FAR4proY1H#9 RV	GAGCACATGCCTCGAGAAAAGAACAAGATGATAACGTGA	Colony PCR/Cloning into pAbAi

1633	FAR4proY1H#10 FW	ATGAATTGAAAAGCTTTCACGTTATCATCTTG TTCTTTT	Cloning into pAbAi
1634	FAR4proY1H#10 RV	GAGCACATGCCTCGAGAGTCAACAAAAGCA GGGGAA	Colony PCR/Cloning into pAbAi
1635	FAR4proY1H#11 FW	ATGAATTGAAAAGCTTTAATGTTCCCCTGCTT TTTGTGA	Cloning into pAbAi
1636	FAR4proY1H#11 RV	GAGCACATGCCTCGAGATACCTAAGTGTTTT GGTTAAACGC	Colony PCR/Cloning into pAbAi
1637	FAR4proY1H#12 FW	ATGAATTGAAAAGCTTCTAAAATTAGAACGC CAACCTTCC	Cloning into pAbAi
1638	FAR4proY1H#12 RV	GAGCACATGCCTCGAGCTCTAGGTAGAGCAA TACGAGAG	Cloning into pAbAi
1639	pGADT7- AD(EcoR1) MYB53 FW	GGAGGCCAGTGAATTCATGGGAAGATCTCCT AGCTCA	Colony PCR/Cloning into pGADT7-AD
1640	pGADT7- ADMYB53(Bam H1) RV	CGAGCTCGATGGATCCTTAAGATTGATAAGA AATGTCTGGA	Cloning into pGADT7-AD
1641	pGADT7- AD(EcoR1) MYB92 FW	GGAGGCCAGTGAATTCATGGGAAGATCTCCT ATCTCTGA	Colony PCR/Cloning into pGADT7-AD
1642	pGADT7- ADMYB92(Bam H1) RV	CGAGCTCGATGGATCCCTAAGGAATGTCGGA AAATATAGAA	Cloning into pGADT7-AD
1643	pGADT7- AD(EcoR1) MYB593 FW	GGAGGCCAGTGAATTCATGGGGAGGTCGCC TTGT	Colony PCR/Cloning into pGADT7-AD
1644	pGADT7- ADMYB93(Bam H1) RV	CGAGCTCGATGGATCCCTAAGATATAACGTT CATGAGGCTT	Cloning into pGADT7-AD
1645	pGADT7- ADMYB53- Nterm(BamH1) RV	CGAGCTCGATGGATCCTTAAGAAGGTTGAAG AAGGTATTGG	Cloning into pGADT7-AD
1646	pGADT7- ADMYB92-	CGAGCTCGATGGATCCCTAGGAAGACGGTTG AAGAAGG	Cloning into pGADT7-AD

	Nterm(BamH1) RV		
1647	pGADT7- ADMYB93- Nterm(BamH1) RV	CGAGCTCGATGGATCCCTAACGTTGGAGATA TTGTAGATTG	Cloning into pGADT7-AD
1648	3' AD RV Seq	AGATGGTGCACGATGCACAG	Sequencing pGADT7-AD
1648	pAbAi FW Seq	GTTCTTATATGTAGCTTTCGACAT	Sequencing pAbAi
1501	LP_CS876373	GCAACAACCAAACCATATTCG	Genotyping T- DNA mutant
1502	RP_CS876373	TTCTCCTCTTCTCCTTCTCCTC	Genotyping T- DNA mutant
1503	LP_SALK201886	TTAATGATGATGGCTCCTTCG	Genotyping T- DNA mutant
1504	RP_SALK201886	CGACTTCACGAGGTATAATTGG	Genotyping T- DNA mutant
1505	LP_CS848035	TTGCTTGTTTCTTGAATTGG	Genotyping T- DNA mutant
1506	RP_CS848035	AATCCACTCAAACCCGAAATC	Genotyping T- DNA mutant
1486	SALK LB-1.3 BP	ATTTTGCCGATTCGGAAC	Genotyping T- DNA mutant
1487	SAIL LB-1 BP	TAGCATCTGAATTCATAACCAATCTCGATAC AC	Genotyping T- DNA mutant

Supplemental Table 4: Constructed vector DNA glycerol stocks

Stock #	Vector	Strain	Use
833	pVKH18-35S:MYB93 CDS	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter Activation Assay
1105	pK7WG2D-35S:MYB53 CDS	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter Activation Assay
1124	pK7WG2D-35S:MYB41 CDS	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter Activation Assay
1170	pMDC32-35S:MYB92 CDS	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter Activation Assay
960	pDONR221: FAR4 promoter full (2300 bp)	<i>E. coli</i> (DH5 α)	Promoter:Gus Expression
964	pDONR221: FAR4 promoter del_1 (245 bp)	<i>E. coli</i> (DH5 α)	Promoter:Gus Expression
966	pDONR221: FAR4 promoter del_2 (546 bp)	<i>E. coli</i> (DH5 α)	Promoter:Gus Expression
969	pDONR221: FAR4 promoter del_3 (1171 bp)	<i>E. coli</i> (DH5 α)	Promoter:Gus Expression
970	pDONR221: FAR4 promoter del_4 (1594 bp)	<i>E. coli</i> (DH5 α)	Promoter:Gus Expression
971	pDONR221: FAR4 promoter del_5 (2061 bp)	<i>E. coli</i> (DH5 α)	Promoter:Gus Expression
1767	pKGWFS7: FAR4 promoter full (2300 bp)	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter:Gus Expression
1768	pKGWFS7: FAR4 promoter del_1 (245 bp)	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter:Gus Expression
1769	pKGWFS7: FAR4 promoter del_2 (546 bp)	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter:Gus Expression
1770	pKGWFS7: FAR4 promoter del_3 (1171 bp)	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter:Gus Expression
1771	pKGWFS7: FAR4 promoter del_4 (1594 bp)	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter:Gus Expression
1772	pKGWFS7: FAR4 promoter del_5 (2061 bp)	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter:Gus Expression
1784	pGREENII0800-PtANR1promoter:LUC	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter Activation Assay
1785	pMDC32-35S:PtMYB134	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter Activation Assay
1786	pMDC32-35S:PtbHLH131	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter Activation Assay

1816	pGREENII0800-FAR4promoter(2300bp):LUC + pSOUP	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter Activation Assay
1817	pGREENII0800-GPAT5promoter(2165 bp):LUC + pSOUP	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter Activation Assay
1906	ASFT promoter in pGREENII0800 (@HindIII/BamH1) + pSOUP	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter Activation Assay
1907	RALPH promoter in pGREENII0800 (@HindIII/BamH1) + pSOUP	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter Activation Assay
1908	HORST promoter in pGREENII0800 (@HindIII/BamH1) + pSOUP	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter Activation Assay
1909	WRKY56 promoter in pGREENII0800 (@HindIII/BamH1) + pSOUP	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter Activation Assay
1915	pKGWDIIG-35S:AtMYB39 CDS	<i>A. tumefaciens</i> (GV3103::pMP90)	Promoter Activation Assay
1917	pGADT7-AD: MYB53 full-length CDS (933 bp)	<i>E. coli</i> (DH5 α)	Y1H Assay (prey)
1918	pGADT7-AD:MYB92 full-length CDS (1037 bp)	<i>E. coli</i> (DH5 α)	Y1H Assay (prey)
1919	pGADT7-AD:MYB93 full-length CDS (1098 bp)	<i>E. coli</i> (DH5 α)	Y1H Assay (prey)
1920	pGADT7-AD:MYB53 N-terminal CDS (581 bp)	<i>E. coli</i> (DH5 α)	Y1H Assay (prey)
1921	pGADT7-AD:MYB92 N-terminal CDS (587 bp)	<i>E. coli</i> (DH5 α)	Y1H Assay (prey)
1922	pGADT7-AD:MYB93 N-terminal CDS (551 bp)	<i>E. coli</i> (DH5 α)	Y1H Assay (prey)
1923	pAbAi-FAR4pro#3 (-2037 bp to -1838 bp from ATG start site)	<i>E. coli</i> (DH5 α)	Y1H Assay (prey)
1924	pAbAi-FAR4pro#4 (-1825 bp to -1826 bp from ATG start site)	<i>E. coli</i> (DH5 α)	Y1H Assay (bait)
1925	pAbAi-FAR4pro#9 (-965 bp to -774 bp from ATG start site)	<i>E. coli</i> (DH5 α)	Y1H Assay (bait)
1926	pAbAi-FAR4pro#10 (-797 bp to -632 bp from ATG start site)	<i>E. coli</i> (DH5 α)	Y1H Assay (bait)

1927	pAbAi-FAR4pro#11 (-658 bp to -563 bp from ATG start site)	<i>E. coli</i> (DH5α)	Y1H Assay (bait)
1928	pAbAi-FAR4pro#12 (-551 bp to -401 bp from ATG start site)	<i>E. coli</i> (DH5α)	Y1H Assay (bait)
1929	pAbAi-FAR4pro#3 (-2037 bp to -1838 bp from ATG start site) – integrated in <i>ura3-52</i> locus	<i>Saccharomyces cerevisiae</i> (Y1H GOLD)	Y1H Assay (bait)
1930	pAbAi-FAR4pro#4 (-1825 bp to -1826 bp from ATG start site) – integrated in <i>ura3-52</i> locus	<i>Saccharomyces cerevisiae</i> (Y1H GOLD)	Y1H Assay (bait)
1931	pAbAi-FAR4pro#9 (-965 bp to -774 bp from ATG start site) – integrated in <i>ura3-52</i> locus	<i>Saccharomyces cerevisiae</i> (Y1H GOLD)	Y1H Assay (bait)
1932	pAbAi-FAR4pro#10 (-797 bp to -632 bp from ATG start site) – integrated in <i>ura3-52</i> locus	<i>Saccharomyces cerevisiae</i> (Y1H GOLD)	Y1H Assay (bait)
1933	pAbAi-FAR4pro#11 (-658 bp to -563 bp from ATG start site) – integrated in <i>ura3-52</i> locus	<i>Saccharomyces cerevisiae</i> (Y1H GOLD)	Y1H Assay (bait)
1934	pAbAi-FAR4pro#12 (-551 bp to -401 bp from ATG start site)	<i>Saccharomyces cerevisiae</i> (Y1H GOLD)	Y1H Assay (bait)

Supplemental Table 5: *FAR4*pro: GUS deletion series images after GUS staining

<i>FAR4</i> Promoter: GUS line	Lateral Root/Leaf	Root Maturation Zone	Root Tip
-2300 bp			
P1
P2
P3
P4
P5

P6
P7
P8
P9
P10
P11

P12
P13
P14
P15
P16
-2061 bp			
P1

P14
P15
P16
P17
-1594			
P1
P2

P3
P4
P5
P6
P7
P8

P9
P10
-1170 bp			
P1
P2
P3
P4

P5
P6
P7
P8
P9
P10

P11
P12
P13
P14
-545 bp			
P1
P2

P9
P10
P11
P12
P13
P14

P15
P16
-244 bp			
P1
P2
P3
P4

P5
P6
P7
P8
P9
P10

P11
P12
P13
P14

Supplemental Table 6: Genes involved in suberin deposition.

Role	Gene Name	Accession Number
Transport	<i>ABCG2</i>	At2g37360
	<i>ABCG6</i>	At5g13580
	<i>ABCG20</i>	At3g53510
Biosynthesis	<i>FACT</i>	At5g63560
	<i>ASFT1</i>	At5g41040
	<i>CYP86A1</i>	At5g58860
	<i>CYP86B1</i>	At5g23190
	<i>FAR1</i>	At5g22500
	<i>FAR4</i>	At3G44540
	<i>FAR5</i>	At3g44550
	<i>GPAT5</i>	At3g11430
	<i>GPAT7</i>	At5g06090
	<i>KCS2</i>	At1g04220
	<i>KCS9</i>	At2g16280
	<i>KCS20</i>	At5g43760
Regulation	<i>MYB9</i>	At5g16770
	<i>MYB41</i>	At4g28110
	<i>MYB53</i>	At5g65230
	<i>MYB92</i>	At5g10280
	<i>MYB93</i>	At1g34670
	<i>MYB107</i>	At3g02940
	<i>MYB39</i>	At4g17785
	<i>NAC046</i>	At3g04060

Supplemental Table 7: Binding of suberin-associated transcription factors to genes involved in suberin deposition.

<u>Target Gene</u>	<u>Accession Number</u>	<u>MYB9 3 DAP</u>	<u>MYB93 amp-DAP</u>	<u>NAC04 6 DAP</u>	<u>NAC046 amp-DAP</u>	<u>MYB107 DAP</u>	<u>MYB107 amp-DAP</u>
ABCG2	At2g37360	yes	yes	yes	yes	yes	yes
ABCG6	At5g13580	yes	no	no	no	yes	no
ABCG20	At3g53510	yes	yes	no	yes	yes	yes
FACT	At5g63560	no	no	yes	yes	no	no
ASFT1	At5g41040	yes	yes	yes	yes	yes	yes
CYP86A1	At5g58860	no	no	yes	yes	no	no
CYP86B1	At5g23190	yes	yes	no	yes	yes	no
FAR1	At5g22500	no	no	yes	yes	yes	yes
FAR4	At3g44540	yes	no	no	yes	no	yes
FAR5	At3g44550	yes	yes	yes	yes	yes	yes
GPAT5	At3g11430	no	yes	no	yes	yes	yes
GPAT7	At5g06090	yes	yes	yes	yes	yes	yes
KCS2	At1g04220	yes	yes	yes	yes	yes	yes
KCS9	At2g16280	no	no	no	no	no	no
KCS20	At5g43760	yes	yes	no	no	yes	no
MYB9	At5g16770	no	no	no	yes	no	no
MYB41	At4g28110	no	no	no	yes	yes	no
MYB53	At5g65230	no	no	no	yes	no	no
MYB92	At5g10280	no	no	no	yes	no	no
MYB93	At1g34670	yes	yes	no	yes	yes	yes
MYB107	At3g02940	no	no	yes	yes	yes	no
MYB39	At4g17785	no	no	no	no	no	no
NAC046	At3g04060	yes	no	no	no	yes	no
bHLH112	At1g61660	no	no	yes	yes	no	no
bHLH111	At1g31050	no	yes	no	yes	no	no
bHLH27	At4g29930	no	no	no	no	yes	no
WRKY56	At1g64000	yes	yes	no	no	yes	yes
BCCP2	At5g15530	no	yes	yes	yes	yes	yes

Supplemental Table 8: Total aliphatic suberin from 12-day old roots of various *Arabidopsis* genotypes

Genotype	Amount (umols/g DW)				Descriptive Stats		
	1	2	3	4	AVG	SD	SE
Wild-type	135.4851	110.256	135.0588	108.0627	122.2157	15.1037	7.55185
<i>wrky56-1</i>	138.0475	147.402	109.0651	116.6497	127.791	17.93112	8.96556
<i>wrky56-2</i>	125.6982	115.6235	121.599	130.5749	123.3739	6.337064	3.168532
<i>wrky56-3</i>	147.968	127.3929	146.5292	112.7744	133.6662	16.79107	8.395533

Supplemental Table 9: Aliphatic suberin compounds of 12-day old roots from various *Arabidopsis* genotypes

Genotype	Amount (umols/g DW)						
	1	2	3	4	Average	SD	SE
	Fatty acids						
Wild-type	40.05805	35.19074	40.21737	30.88326	36.58735	4.461311	2.230655
<i>wrky56-1</i>	40.10494	42.42527	27.62512	36.63505	36.69759	6.499484	3.249742
<i>wrky56-2</i>	34.47179	25.67284	31.21961	34.82699	31.54781	4.239722	2.119861
<i>wrky56-3</i>	33.41222	30.63161	35.66651	28.50859	32.05473	3.135183	1.567591
	Dicarboxylic fatty acids						
Wild-type	33.88699	28.27555	37.75631	28.17979	32.02466	4.660435	2.330217
<i>wrky56-1</i>	36.50888	36.91379	24.50081	33.04844	32.74298	5.76207	2.881035
<i>wrky56-2</i>	34.66045	28.43988	31.37269	31.83965	31.57817	2.546889	1.273445
<i>wrky56-3</i>	34.02537	31.72053	36.51602	29.4541	32.929	3.033374	1.516687
	ω-hydroxy fatty acids						
Wild-type	45.43541	35.36471	43.5411	38.28031	40.65538	4.647877	2.323938
<i>wrky56-1</i>	50.46328	55.40454	47.90528	46.22129	49.9986	4.003754	2.001877
<i>wrky56-2</i>	40.79879	41.98128	44.60124	46.83396	43.55382	2.703053	1.351527
<i>wrky56-3</i>	52.30945	47.35669	50.90661	43.00105	48.39345	4.155474	2.077737
	Fatty alcohols						
Wild-type	9.004422	6.660086	9.189354	7.073674	7.981884	1.300713	0.650356
<i>wrky56-1</i>	7.682106	8.860068	6.362956	6.301549	7.301669	1.218573	0.609287
<i>wrky56-2</i>	6.837364	5.597236	6.903145	6.88476	6.555626	0.639528	0.319764
<i>wrky56-3</i>	9.913942	7.135468	10.16468	7.289245	8.625833	1.636553	0.818277
	Ferulate						
Wild-type	7.10028	4.764895	4.354657	3.64568	4.966378	1.495836	0.747918
<i>wrky56-1</i>	3.288271	3.798284	3.805565	3.499936	3.598014	0.250829	0.125414
<i>wrky56-2</i>	4.174559	8.949454	3.202337	6.102895	5.607311	2.533259	1.266629
<i>wrky56-3</i>	17.32927	3.773134	13.49141	4.808553	9.850592	6.621777	3.310889

Cis-element Map

< FAR4 Promoter (2300 bp) - 2300 nucleotides

MYB AC-rich = **blue** (ACCWAMY and ACCWHH)

MYB-core = **baby blue** (YAACNG or YNGTTR)

WRKY W-box = **pink** (TGAC or TTGACY)

WRKY WT-box = **purple** (GGACTTTC)

WRKY WK-box = **purple** (TTTTCCAC)

bHLH E-box = **yellow** (CANNIG or specific G-box CACGTG)

bHLH non E-box = **red** (NRCGTG)

bHLH N-box = **red** (CACGRG)

bHLH CGC-box = **red** (GGKCKRWC)

NAC-core = **green** (CGTR) → palindromic (CTTN₇AAG) and (CGTN₇ACG)... manual check

NAC-ZF = **dark green** (CCGCC)

NAC cluster 3 = **dark green** (TTVCTT)

CAAT-box = **teal** (CCAAT)

TATA-box = **gold** (TATAWAW)

* = nucleotides shared between adjacent motifs

Top strand = + = 5' → 3'

Bottom strand = - 3' → 5'

-2300 bp

FL

ggaacctatgtccgaactccgaattggcttgatatgtccgatgcatttagacaaaaatcaaattggtttgaatat
ccttggtacaggcttgaggcttaaccgaactatacaggcta**cgta**aactctgttttagtttaacaaaacttata

catcgctggcttcttcatattttgttggttcaaaagaaaagttggttggtgtacagtgaaatatttgatggccaca
gtagcgaccgaagaagtataaaaaaacaagaagttttcttttcaacaaacaacatgtcattataaacataccggtgt

-2150 bp

atcagatttagtttgatattatcaattattcaaatat**atgc**agaattttcttcaagttacatacatggcttgat
tagtctaaatcaaactataatagtttaataagtttaaatcgtctttaaagaagttcaatgtatgtaccgaacata

del 5

gcatattcttc**cgtagcagtt**taacc**ccg**acataagggatagggcagcggtgtatgg**acctca**tatttaattatt
cgtataagaaggcatcgtcaagattggggctgtattccctatcccgctcgccacatacctggagtataaaatataa

-2000 bp

aaaaaat**tataaaaaaattg**ttagtgg**tataaaa**tactctcg**aaaaatataaat**acattttttttgtgcacaataa
tttttaatatattttttaacaa**tcaccata**ttttatgagagcttttatattttatgtaaaaaaaa**cagtg**ttatt

tacatatatttatatttgcaaatatagaattttattttaataacaagactaaaatcttctttttttccctaaggct
atgt**tataaatat**aaacgttttatatctttaaataaatttt**attgtt**ctgattttagaagaaaaaaagggtattccga

cttttta**cagttggg**ccggggctgat**cagtt**cgctattgaagtata**catgt**caaagaaaatgaaggagaat**aaac**
gaaaaat**gtcaac**ccggccccgactagtcaagcgataactt**catatg**ta**cagtt**cttct**taactt**cctcttatttg

catcgtatttaattaaatcgatttgactaatgagacaacttaattcagctttaatatagtttaaat**cgta**catg
gtagcataatttaatttagctaaactgattact**ctgtt**gaattaagtcgaaattatatcaaatttaaaagcatgtac

-1700 bp

AC-rich (x2)

accaggtaatcaaattttctaa**caaat**ggaacaatatcagatagg**gtaaactgttac****catctg**tgtaactttgc
tggtccattagttttaaagatttt**gtttac**cttggttatagtcctatcc**catttgac**aatggtagacacattgaaacg

Del 4

aaattagtaagtctaaattataagaaacgatgacttgtacttctaaaactccagcatatcatataaaaattttgag
tttaatcattcagatttaataattctttgtactgaacatgaagattttgaggtcgtagtagtatattttaaaactc

-1550 bp

gtttgagacatttctataagttcagatttaattcttctctaaacgtatgagggttaggtattttctgtcgtttacca
caaaactctgtaaagatattcaagtcctaaattaagaaggattgcatactccaatccataaaagacagcgaaatggt
AC-rich (x2)

AC-rich (x5)

aaaaatttgacgtttcccatagcttttagcttgttatgtcattgttcataatttatgaccaaccaaacctaaga
tttttaactgcaggggtatgcataaatcgaacaatacagtaacaaacgtattaaatactgggttggtttggattct

ccaaatcagggttttttagatagtccttttttctgttgaaattataagatgttttggttgattggcatagattaca
ggttttagtcacaaaatctatcaggaaaaaaggaaactttaataattctacaaaaccaacataaccgtagtctaagt

aaaattactccccccatttttagattagctgtcgttgaagatttttacagaataataaaaaaacattcaatttttc
tttaaatgaggggggtaaaatctaatacgacagcaacttctaaaaatgtcttattatttttttgtaagttaaaaag

-1250 bp

tgttttaccctaattaatgtattgtttatatttgatatcatcaataaacgttttaaaaatgttgataaaattgga
acaaaatgggattaattacataacaataataaactatagtagtattatgcataatttttacaactattttaacct

del 3

aactttgtcaaacatctgcattgtaatatataatctaaaaaaaatgatttcttatattctatcaaatatggctaa
ttgaaacagttttagacgtaacatttatatttagattttttttactaaagaatataagatagtttataccgatt

-1100 bp

ctatacacatataatttgggctgagctgtcatctttcgacatgaaattactccgaattatacataatttgcaga
gatatgtgtatattaaacccgactcgacagtagaaagctgtactttaatgaggcttaatatgtattaaacggtct

gaagttatgttacataacaacactcatattcaaaagaaaactagatgtataatgtattagctattattttaagtaa
cttcaatacaatgtattgttgtagtataagttttcttttgatctacataattacataatcgataataaaatcatt

-950 bp

TSS?

cactaacaactaaataaaaatagaatggatgaaactataaaaaaattaagaattattattgaatgagtatattttt
gtgattgttgattttatttttatcttacctacatttgatatttttttaattcttaataataaacttactcatataaaaaa

tctatttttggtcaaacgtcgagggtggagtttaagaaaatcgtgattctaaaatagtgcaataatgaaatattaaa
agataaaaccagtttgcagctccacctcaattcttttagcactaagattttatcacggttaacttttataattt

-800 bp

aaatcacggttatcatcttgttcttttattttccactattttctcttgaaatttaaatgaaatatgttatttcca
tttagtgcaatagtagaacaagaaaataaaagggtgataaaagagaactttaaattaactttatacaataaaaagg

cDNA end

aaatgttggttacaaccgatttactatctcaataatatgttcttttgtgattttaatttttgattatttaattgttc
tttacaacaatgttggctaaatgatagagtattttatacaagaaaacactaaaattaaaaacataataattacaag

-650 bp MYBcore (x2) /E-box

ccctgctttttgttgactaaacaactcgttactcgtttagatataatcaccgttgagaaaaagcgtttaaccaaa
gggacgaaaaaacaactgatttgttgagcaatgagcaatctatatataagtgcactcttttttcgcaaattgggt

Del_2

aacacttaggtatattgaagagttctaaaattagaacgccaaccttccaaatcaacacacctacgacaattctag
ttgtgaatccatataaacttctcaagattttaatcttgcggttggaaggttagttgtgtggaatgctgttaagatc

-500 bp

tcctacaacatagatccttctagcaaacccacaaggaaacttcttgtggaaaacactaacactatcctcactcagt
aggatgttgtatctaggaagatcgtttgggtgttcccttgaagaacaccttttgtgattgtgataggagtgaagtc

tactctcgattattgctctacctagagtttctaatctgctatgtataaaaaattcttacatacttattcttttttt
atgagagcataacgagatggatctcaaagattaagacgatacaaatatttttaagaatgtatgaataagaaaaaaa

-350 bp

tctttctaaacattccattcggttttaaaagctaggcccacgattagtagttaaaaatattttaatttttaaaaaata
agaaagatttgaaggtaagcaaaattttcgatccgggtgcataatcatcaattttataaaaattaaaaatttttat

del_1

tgggttacatattttttattcttttttagagctccgagatttcagagcatgttctaatggatatgtgatatttaa
acccaatgtataaaaaataagaaaaatctcgaggctctaaagtctcgtaacaagattaacctatacattataaatt

-200 bp

tcagaagtagttttaattattgttttttttttataaaaagaaaatgtaggttaggtttacgtacaagtagtagatatg
agtcttcatcaaaattataacaaaaaaaataattttcttttaacatccatccaaatgcattgttcatcatctatac

5' UTR/flcDNA start

atgggtcaacaaaacgggtcaacatttacgggtctatatatattcggtgctcatcgccgaagggtgttatgggtca
taccgagttgttttgcacagttgtaaatgcccagatatatataagcacggagtagcgggttccacaataccaagt

E-box

-50 bp

aaacaatctatcgtaacttagttattaattggatagatataagtttcttcaATG
tttgttagatagcatgaatcaataattaacctatctatattcaaagaagtTAC

50 bp last line, 75 bp all other lines

0 tandem repeats and 0 CpNpG islands.

Supplemental Figure 1: Map of the *FAR4* 5' promoter upstream region with specific motifs

Supplemental Figure 2: Map of the T-DNA region in pGREENII 0800-LUC

Supplemental Figure 3: Vector map of pSOUP

Supplemental Figure 4: Vector map of pDONR221

Supplemental Figure 5: Vector map of pKGWFS7

Figure 3.1 Schematic representation of pVKH18-EN6 binary vector, used for transient expression of cardosin A and its mutated versions in tobacco leaves. En6 - 35S CaMV promoter + 6x enhancer; Hyg^r - hygromycin resistance gene; LB - left border, RB - right border; Tnos - nopaline synthase terminator. Adapted from Batoko et al. (2000).

Supplemental Figure 8: Vector map of pVKH18

Supplemental Figure 9: Vector map of pAbAi

Supplemental Figure 10: Vector map of pGADT7-AD