The role of transcription factor MYB53 from *Arabidopsis thaliana* in the regulated production of suberin

by

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Abstract

Suberin is a cell wall-associated polymer that is deposited in diverse plant tissues including root exodermis and endodermis, aerial and underground periderms, and seed coats under both normal and stressful conditions. Suberin plays important roles in protecting plants against various stressors but the molecular mechanisms governing the regulated deposition of suberin are currently unclear. I provide evidence here that AtMYB53, AtMYB92, and AtMYB93 of the MYB-type transcription factor family are important regulators of suberin in root endodermis under non-stress conditions. I first characterized an Arabidopsis steroid-inducible line and found that suberin can be rapidly induced in both roots and leaves upon overexpression of *MYB53*. A suite of suberin biosynthetic genes was positively regulated at the transcriptional level after *MYB53* overexpression. I also generated a collection of loss-of-function mutants of *MYB53/MYB92/MYB93,* which exhibited major reductions of suberin in the endodermis of young roots in comparison to wild-type. The transcripts of all suberin biosynthetic genes tested were down-regulated in the mutants. The identification of master regulators of suberin may provide the means to generate crops that are more stress resistant via enhancement of their suberized cell walls.

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

ABC transporter	ATP-binding cassette transporter
AHCs	Alkyl hydroxycinnamates
ASFT	Aliphatic suberin feruloyl transferase
At	*Arabidopsis thaliana*
bHLH	Basic helix-loop-helix
C	Carbon
C16:0	Palmitic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C20:0	Arachidic acid
C22:0	Behenic acid
C24:0	Lignoceric acid
$Ca(NO_3)_2$	Calcium nitrate
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin ImmunoPrecipitation
CHIP-seq	Chromatin ImmunoPrecipitation sequencing
CoA	Coenzyme A
Ct	Threshold Cycle
CYP	Cytochrome P450
DEX	Dexamethasone
DCA	Dicarboxylic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediamine tetraacetic Acid
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
FA	Fatty acid
FACT	Fatty Alcohol:Caffeoyl-CoA Caffeoyl Transferase
FAE	Fatty acid elongase
FAR	Fatty acyl reductase
FHT	omega-hydroxyacid - Fatty alcohol hydroxycinnamoyl transferase
g	Gram

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GPAT5	Glycerol-3-phosphate acyltransferase 5
GUS	β-glucuronidase
KCS	β-ketoacyl-CoA synthase
KH_2PO_4	Potassium dihydrogen phosphate
KNO_3	Potassium nitrate
L	Liter
LACS	Long-Chain Acyl-CoA Synthetase
mg	Milligram
$MgSO_4$	Magnesium sulfate
ml	Milliliter
m	Meter
mm	Millimeter
mM	Millimole
MYB	*v-myb* myeloblastosis viral oncogene homolog
NaCl	Sodium chloride
OH	Alcohol
PCR	Polymerase chain reaction
PP2A	Protein phosphatase 2A
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR
s	Second
SELEX	Systematic evolution of ligands by exponential enrichment
TEM	Transmission electron microscopy
TF	Transcription factor
Tris- HCl	Tris(Hydroxymethyl) aminomethane- hydrochloride
µg	Microgram
µM	Micromole
µl	Microliter
ω-OH	ω-hydroxy fatty acid
WT	Wild-type
VLCFA	Very-long-chain fatty acid

Chapter 1: General Introduction

1.1 Sites of suberin deposition and subcellular localization

Suberin is a plant cell wall-associated and lipid-based polymer that is deposited at interfaces where a barrier is needed to modulate water and solute passage in normal development (Schreiber, 2010; Beisson *et al.*, 2012; Franke *et al.*, 2012). It is also formed to protect against environmental stresses such as high salinity, wounding, and pathogen ingress (Kolattukudy, 1984; Franke and Schreiber, 2007). Suberin is formed in cell walls of external root tissues, such as the epidermis (also called "rhizodermis") of young roots, root periderms (which develop from the pericycle and replaces endodermis, cortex and epidermis during the secondary growth of mature roots), and tuber periderms (e.g. potato skins) (Nawrath *et al*., 2013). Suberin also forms in the cell walls of internal root tissues such as the exodermis and endodermis, which are the outermost and innermost layers of the root cortex, respectively, of young roots (Vishwanath *et al.*, 2015). A well-known example of highly suberized tissue is the cork layer in tree bark, a layer that is expanded in cork oak trees, where suberin can make up to 50% of the chemical composition (Pereira, 1988; Soler *et al*., 2007). Additionally, suberin is deposited in the cell walls of bundle sheaths of C4 plants (e.g. maize and sugarcane) (Beisson *et al*., 2007) and in the chalazae micropyle region of seeds and the outer integument of mature seed coats (Espelie *et al*., 1980; Molina *et al*., 2007). Apart from these suberization patterns under

normal development, suberized tissue can also be formed after mechanical wounding, organ abscission, or due to damage by insects or pathogens (Vandoorn and Stead, 1997). The wide spectrum of suberin elicitation, including in normally non-suberized tissues, indicates that most plant cells can develop into a suberized cell. Therefore, suberin is deposited wherever and whenever it is necessary for plants to build up a hydrophobic barrier for protective purposes (Ranathunge *et al.*, 2011). Suberin localization at the tissue level is typically visualized by microscopy using the suberin-specific fluorescent stain fluorol yellow 088 (Figure 1.1-a, b).

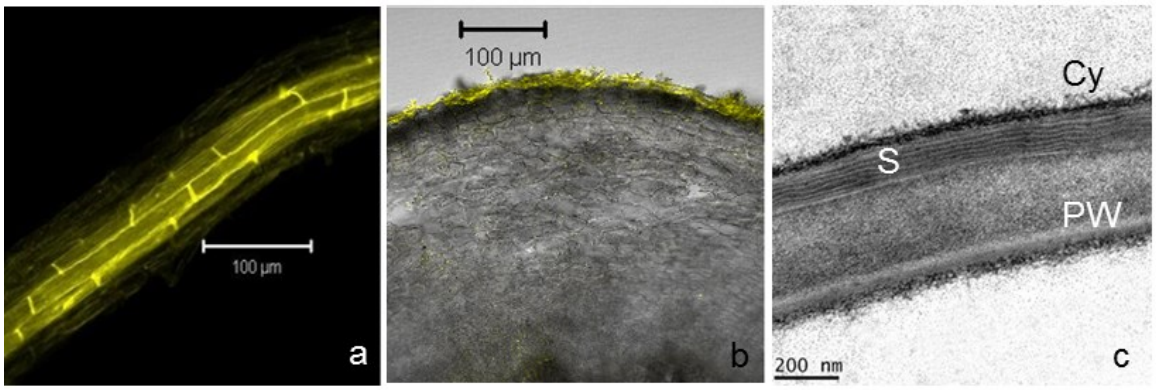


Figure 1.1. Histochemical observation of suberin distribution in various plant tissues.
(a) Arabidopsis young roots with suberin lamellae in endodermis stained with fluorol yellow. (b) Arabidopsis mature roots with suberin in periderm stained with fluorol yellow. (c) Transmission electron microscopy images of Arabidopsis suberized root endodermis cells showing suberin lamellae (alternating light and dark bands). PW, primary cell wall; S, suberin; Cy, cytoplasm. Micrographs were taken by Nayana de Silva, Rowland lab.

At the subcellular level, suberin is typically located on the inner face of primary cell walls adjacent to the plasma membrane and occurs in three forms (Ranathunge *et al*., 2011). In the first form, suberin accumulates in the inter-microfibrillar channels of the young root epidermis and this type of suberin is called "diffuse suberin" (Peterson *et al*., 1998; Nawrath *et al*., 2013). In the second form, suberin is found in lignin-containing Casparian strips, which is localized in the radial and transverse directions of primary cell walls of the root endodermis and exodermis, thus sealing the spaces between adjacent endodermal or exodermal cells (Enstone *et al.*, 2003; Nawrath *et al*., 2013; Vishwanath *et al*., 2015). Casparian strips function as barriers impeding apoplastic movement of water, ion and gas between the outer (cortex) and inner (central stele) cell layers (Peterson *et al*., 1998; Alassimone *et al.*, 2009). In the third form found in root endodermis and exodermis, suberin is deposited as a sheet-like structure on the inner surface of primary cell walls. In observations using transmission electron microscopy (TEM), this "sheet" structure is displayed as alternating light and dark bands and these "suberin lamellae" are typically regarded as an indicator of suberin appearance (Figure 1.1-c).

In the exodermis and endodermis of young roots, both Casparian strips and suberin lamellae are important cell wall-based apoplastic and transcellular barriers in the root endodermis (Nawrath *et al*., 2013). In the first stage of endodermal development, the Casparian strip is formed in the radial and transverse directions of primary cell walls.

Later, during the transition between the first and second developmental stages, suberin deposition begins sporadically in a “patch”-like fashion and suberin lamellae is finally formed all around the endodermal cell walls during the second developmental stage (Andersen *et al.*, 2015; Doblas *et al.*, 2017).

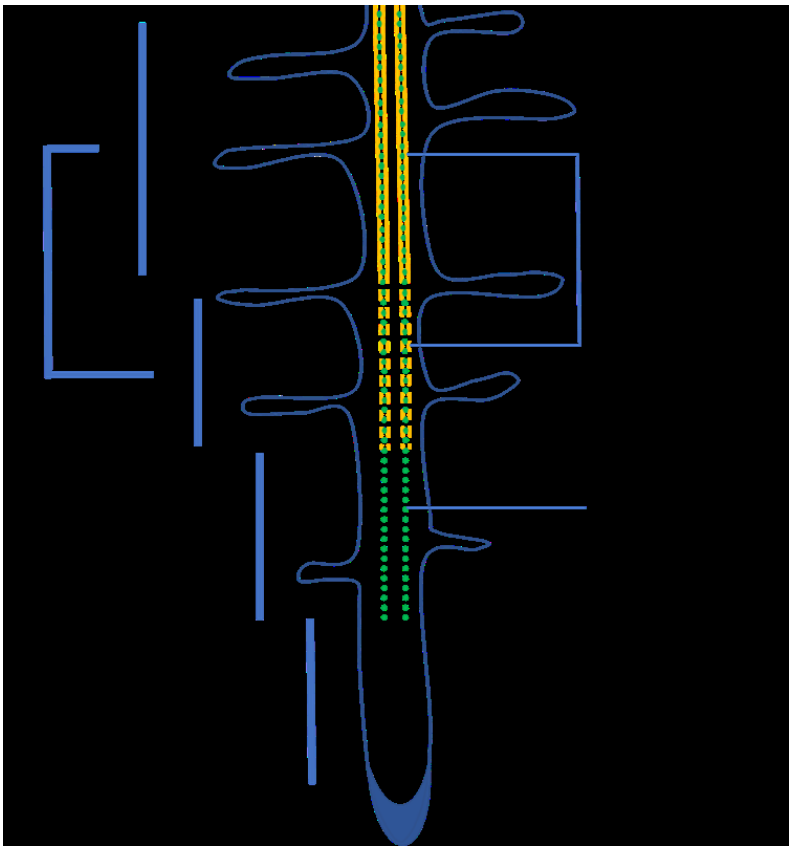


Figure 1.2. Schematic representation of endodermal differentiation in roots. Three zones are highlighted as undifferentiated zone, non-suberized zone and suberized zone. Within the suberized zone, a patchy zone (stage 1) is where only some cells are suberized and a continuous zone (stage 2) is where nearly all cells are suberized with the exception of some passage cells.

Additionally, in the periderms of mature roots and stems (barks), suberin lamellae are deposited in the primary and secondary cell walls as poly-sheets of alternative light and

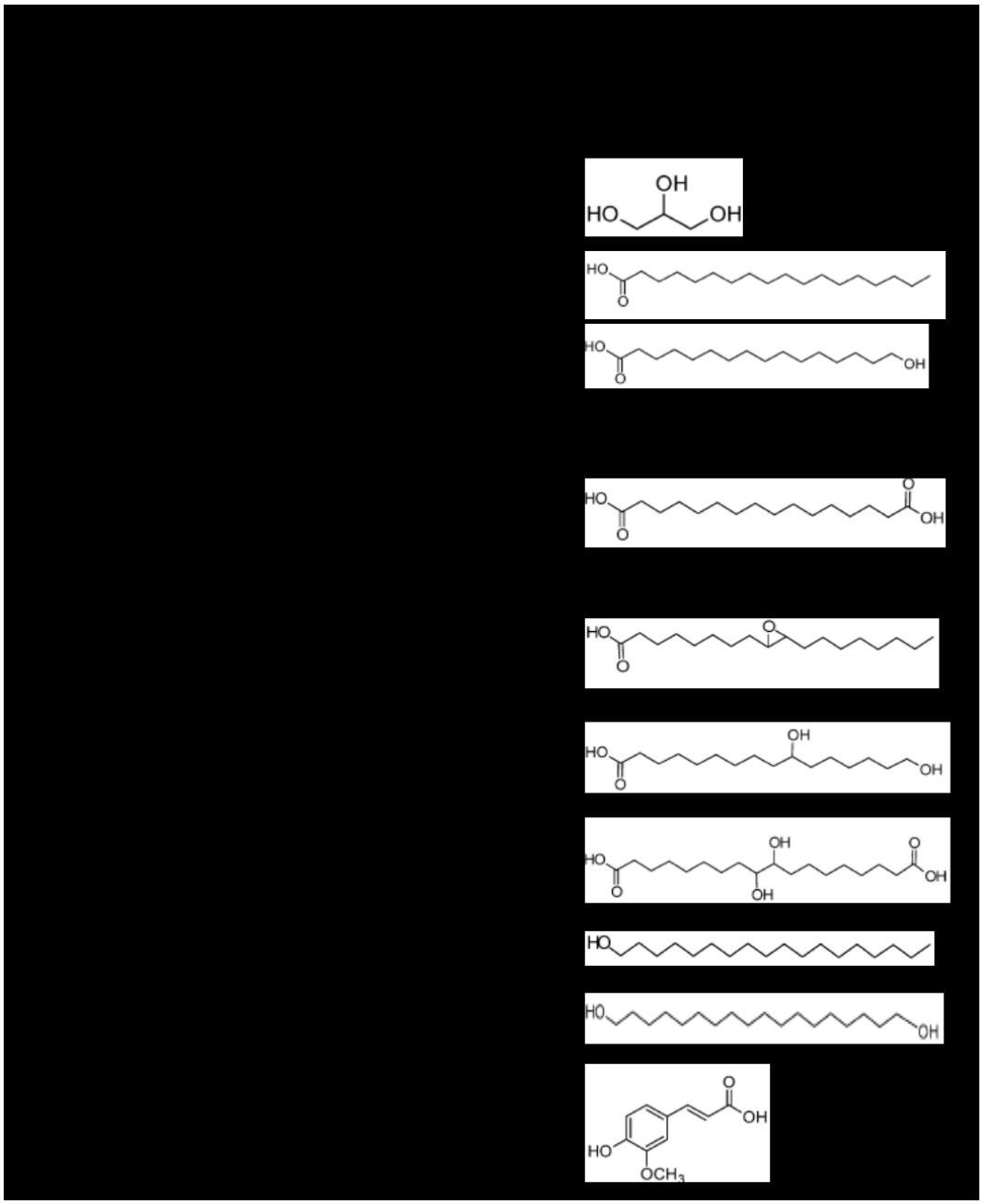
dark bands (Graça, 2015). According to the pioneering TEM work of Sitte (1962), 30 to 60 light and dark bands of suberin lamellae could be counted across the primary and secondary cell walls in cork tissue.

1.2 Suberin chemical composition

Suberin is a chemically complex hetero-polyester mostly composed of glycerol, omega-hydroxy and dicarboxylic fatty acids, primary fatty alcohols and ferulate (Franke and Schreiber, 2007; Pollard *et al*., 2008; Graça, 2015). Compositional analysis of suberin monomers is usually conducted by chromatography techniques (mainly gas chromatography (GC) coupled with mass spectrometry (MS)) (Ranathunge *et al.*, 2011). Suberin aliphatic monomers include long-chain (C16 and C18) and very-long-chain (≥C20) monomers, such as α,ω-dicarboxylic acids, ω-hydroxy fatty acids, mid-chain hydroxy or epoxy fatty acids, unsubstituted fatty acids, and primary fatty alcohols (Table 1.1). Glycerol plays a role as the basic bridge to link the various types of fatty acids by esterification of at least one of its hydroxyl groups with the carboxyl groups of the fatty acyl chains (Kolattukudy, 1984; Graça and Santos, 2007; Andersen *et al*., 2015). In addition, hydroxycinnamic acids are the typical components of aliphatic suberin, with ferulate being the most common (Table 1.1). Ferulic acids are mostly esterified with ω-hydroxy fatty acids in the polymer (Pollard *et al*., 2008; Graça, 2015).

Table 1.1 Chemical structures of common suberin monomers with typical carbon lengths and degrees of saturation.

(Table modified from Pollard *et al.*, 2008)



It's important to note that the total and relative amounts of suberin monomers are highly variable depending on developmental stage, tissue, and plant species (Zeier and Schreiber, 1998; Ranathunge *et al*., 2011; Vishwanath *et al*., 2015). For example, suberin monomers from cork oak (*Quercus suber*) and Douglas fir (*Pseudotsuga menziesii*) bark are mainly α,ω-dicarboxylic acids (Graça and Pereira, 2000a), whereas suberin from potato (*Solanum tuberosum*) tuber periderm (Graça and Pereira, 2000b) and Arabidopsis (*Arabidopsis thaliana*) roots (Franke *et al*., 2005) are characterized by similar amounts of ω-hydroxy fatty acids and α,ω-dicarboxylic fatty acids. Even within one species there is variability in suberin composition, such as Arabidopsis where suberin from roots is predominated by C16, 18:1, and C22 monomers (Franke *et al*., 2005), whereas suberin in seed coat is more highly enriched in C24 monomers (Molina *et al*. 2006).

Additionally, the suberin polymer is embedded with soluble lipids (unpolymerized aliphatics) called suberin-associated waxes (Vishwanath *et al*., 2015), which are considered to be the major contributors to the barrier against water diffusion across suberized cell walls (Soliday *et al.,* 1979; Espelie *et al*., 1980). The waxes are solvent-extractable by brief (1-2 min) immersion of tissues in chloroform (Li *et al*., 2007; Molina *et al*., 2009; Kosma *et al*., 2012; Vishwanath *et al*., 2013) or by extensive solvent extraction of isolated periderms (Schreiber *et al.*, 2005; Serra *et al*., 2010; Delude *et al.*,

2016). In Arabidopsis roots, some aliphatic waxes are suberin-associated and thus have characteristics common to some of the monomers released upon depolymerization of root suberin (Franke *et al.,* 2006; Beisson *et al.,* 2007). These suberin-associated waxes include saturated fatty acids (commonly C16–C22), 18:0-22:0 fatty alcohols and alkyl hydroxycinnamate (AHC) esters (Li *et al*., 2007; Pollard *et al*., 2008). The AHC esters are comprised of phenylpropanoids, typically coumaric, ferulic, or caffeic acids, esterified to fatty alcohols, and up to 80% of the primary fatty alcohols in Arabidopsis roots exist in the form of AHC esters in suberin-associated waxes and alkyl caffeates are the predominant type (Kosma *et al.,* 2012; Vishwanath *et al*., 2013; Delude *et al*., 2016). Meanwhile, Arabidopsis root waxes also include alkanes (typically C29 and C31) and their mid-chain oxidized (keto or hydroxyl) derivatives, monoacylglycerols (typically with C20 and C22 acyl chains) and sterols (Kosma *et al.,* 2012; Kosma *et al.,* 2015).

1.3 Suberin structure

Bernards (2002) proposed the following about suberin structure and its definition: 1) ferulic acids should be included as a component in suberin poly-aliphatic domain (SPAD) when they are esterified with fatty acids and fatty alcohols; 2) a poly-aromatic part made up of hydroxycinnamic acids and monolignols, which are located in primary cell walls, should be considered as a suberin polyphenolic domain (SPPD) and

this polyphenolic domain of suberin is covalently linked with the suberin poly-aliphatic domain (Figure 1.2). However, this definition and the relationship between the polyaliphatic and polyaromatic domains are much debated. According to some more recent models (Graça, 2015), the word "suberin" should only be referred to as the aliphatic polyester (Figure 1.3). Several reasons support this argument. Firstly, suberin can be de-polymerized using base- or acid-catalyzed trans-methylation to yield a variety of aliphatic monomers, including some ferulate, but this does not yield the polyaromatic monomers. Secondly, the polyaromatics are distinct chemically and structurally and also spatially separated from the aliphatic suberin in suberized cell walls. Lastly, the aliphatic suberin has a defined macromolecular structure that is independent from polyaromatics: 1) glycerol molecules link in succession with α,ω-dicarboxylic acids to form the core backbone of the suberin polymer; 2) 18:1 ω-hydroxyacids and α,ω- dicarboxylic acids are dominant components of suberins across many plant species; 3) from de-polymerized suberin polymers, all ω-hydroxy fatty acid monomers are esterified with ferulic acids though their ω-hydroxyl groups (Graça and Pereira, 1998, 1999, 2000b). It may even be possible to consider the polyaromatic domain simply as lignin (Graça, 2015). Similarly, Geldner (2013) also stated that although lignin (composed of phenols) can be additionally modified with aliphatic suberin in Casparian strips, lignin is not an integral part of suberin. As all the discussions above interpret, there are obvious differences between

aliphatic suberin and the poly-aromatics, but it doesn't exclude that there are extensive contacts, including covalent linkages, between these two types of polymers, which is why Bernards considers the two together functionally as "suberin". This debate remains unresolved.

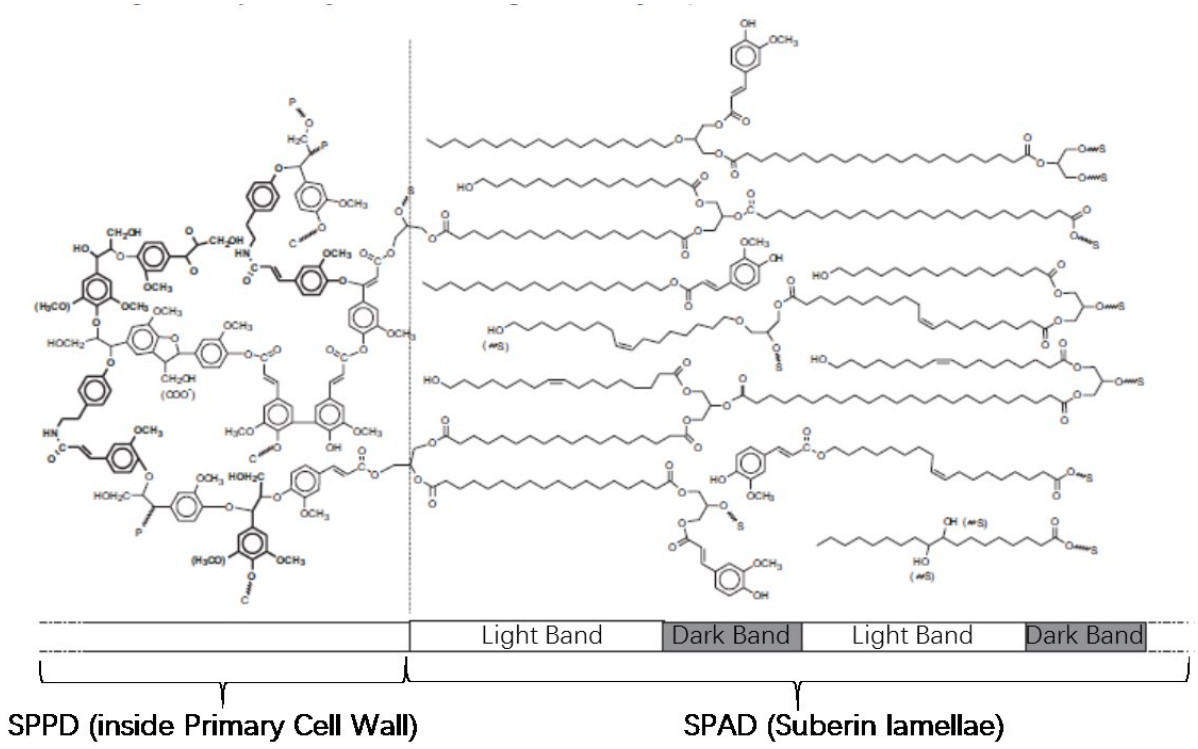


Figure 1.3. Model of suberin structure in suberized potato cell walls as proposed by Bernards (2002).
The suberin poly-phenolic domain (SPPD) is attached to the carbohydrates in the primary cell wall, and the glycerol based suberin poly-aliphatic domain (SPAD) is located between the cell wall and plasma membrane. Light bands represent aliphatics and dark bands represent phenolics. Figure is modified from Bernards (2002).

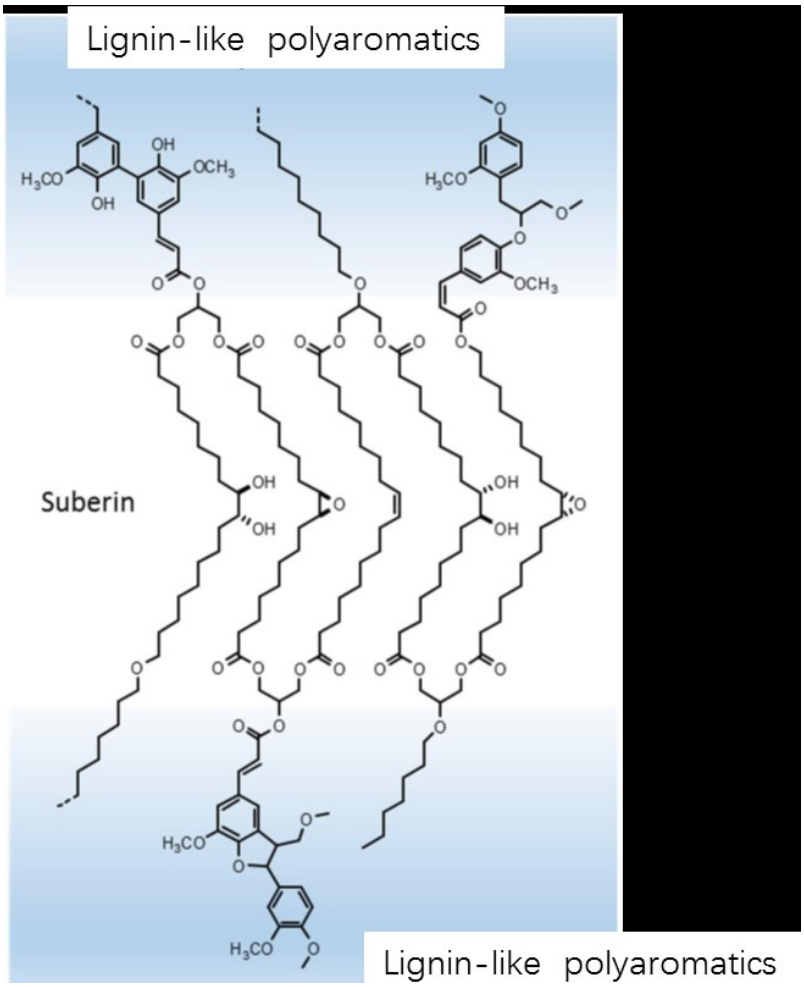


Figure 1.4. Model of suberin structure in the context of suberized cell walls.
The core suberin polymer is presented as a regularly packed poly-acylglycerol molecule, which make up the translucent lamellae. This suberin polyester is covalently linked through esterification to ferulic acid to the neighboring lignin-like polyaromatics, which accounts for the dark lamellae. Figure was modified from Graça (2015).

1.4 Functions of suberin

Water and solutes follow three potential pathways while being transferred from the soil to the central vasculature: (1) apoplastic pathway, via the extracellular space; (2) symplastic pathway, via cytosolic connections called plasmodesmata; (3) transcellular pathway, via polarized influx and efflux transporters and diffusion gradients through the plasma membrane (Doblas *et al*., 2017). Experimentally, the symplastic pathway and transcellular pathway cannot be completely separated so they are collectively called the cell-to-cell pathway (Steudle and Peterson, 1998). It has been reported that the suberized cell walls serve as barriers reducing the uncontrolled transportation of water and dissolved ions (Franke and Schreiber, 2007; Ranathunge *et al*., 2011). For example, an Arabidopsis mutant *cyp86a1/horst* with 60% decreased amount of suberin has a reduced barrier to the radial flow of water and NaCl in the root (Ranathunge and Schreiber, 2011). It is unclear, however, whether suberin is functioning to restrict movement via the apoplastic pathway, the transcellular pathway, or both (Steudle and Peterson, 1998). Meanwhile, the Casparian band restricts apoplastic movement, but not transcellular movement because it is deposited in a polar fashion as a ring and seals the adjacent endodermal cells (like a tight junction in animal epithelial cells) (Barberon and Geldner, 2014). Suberin lamellae, on the other hand, are deposited all around the endodermal cells but on the inner face of the cell walls only (not sealing adjacent cells), thus probably

restricting mostly transcellular movement. Additionally, since the apoplastic / transcellular blockage offered by Casparian strips and suberin lamallae is non-directional, these barriers prevent the backflow of water and ions accumulated in the stele. This is believed to enable plants to build up root pressure (Enstone *et al.*, 2003).

Although the protective functions of Casparian strips and suberin have been reported by many experiments (as mentioned above), it does not mean that these are perfect barriers blocking passage of all solutes. For example, apoplastic bypass flow of the plant hormone abscisic acid (ABA) in maize (*Zea mays*) roots (Schraut *et al*., 2004) as well as the nutritional ion Ca^{2+} in Arabidopsis (*Arabidopsis thaliana*) roots (White, 2001) still occur radially across roots. One possible reason for this is that the suberin lamellae deposition is not completely continuous, but is interspersed with non-suberized passage cells (Waduwara *et al*., 2008).

Suberin also plays an important role in protecting plants from various abiotic and biotic stresses, including exposure to heavy metal, high salts, low oxygen, harmful pathogen attacks and mechanical wounding. For example, suberin can be deposited to block uptake of heavy metals, such as Hg^{2+}, Rb^{+} and Cd^{2+} (Barrowclough *et al.*, 2000; Clarkson *et al*., 1987; Schreiber *et al.,* 1999). Also, suberin deposition is induced by salt stress and accumulation of suberin in roots has been shown to negatively correlate with sodium translocation to aerial organs (Krishnamurthy *et al.*, 2011). In wetland

environments, there is insufficient amounts of oxygen dissolved in water and where soil microorganisms consume large amount of oxygen, and in these conditions rice (*Oryza sativa*) produces more root suberin to impede radial oxygen loss from the roots to the environment (Schreiber *et al*., 2005; Kotula *et al*., 2009). Moreover, another report using *Phragmites australis* (common reed) showed that its resistance to radial oxygen loss was correlated with root suberin content but not with the amount of root lignin (Soukup *et al*., 2007). Although suberin deposition cannot completely stop pathogen invasion, it has been shown that a resistant soybean line with highly suberized cell walls delayed the colonization of *Phytophthora sojae* into epidermis by 2-3 hours, and induced extra aliphatic suberin up to 4 days earlier than a susceptible soybean line (Ranathunge *et al.*, 2008). Additionally, aliphatic suberin and associated polyaromatics may function differently in pathogen defenses. For example, from infection studies using potato wound periderm, it was found that the polyaromatic domain was important for resistance against *Erwinia carotovora* subsp. *carotovora* (a bacterium), while the polyaliphatic domain was important for resistance against *Fusarium sambucinum* (a fungus) (Lulai and Corsini, 1998).

1.5 Suberin biosynthesis and deposition

The production of suberin involves the coordinated production of chemically

diverse compounds including aliphatic, phenolic and glycerol monomers, as well as subsequent transport to the cell wall and polymerization. The mechanism of suberin biosynthesis was first investigated using classical biochemical methods (Kolattukudy, 1981; Bernards, 2002; Franke and Schreiber, 2007). These biochemical studies largely focused on ω-hydroxylation of fatty acids and the subsequent oxidation of ω-hydroxyl fatty acids to α,ω-dicarboxylic fatty acids. However, this approach, involving protein extraction and *in vitro* assays, is difficult because the enzymes involved in biosynthesis of suberin monomers are associated with membranes and / or are likely composed of enzyme complexes (Vishwanath *et al*., 2015).

Recently, the most successful studies in suberin biosynthesis have been achieved using reverse genetic approaches (Vishwanath *et al*., 2015). A network of enzymes functioning in the production of oxygenated fatty acids, primary fatty alcohols, phenolics and acyl-glycerols of suberin have been revealed in the past 10 years, as described in detail below and summarized in Figure 1.4.

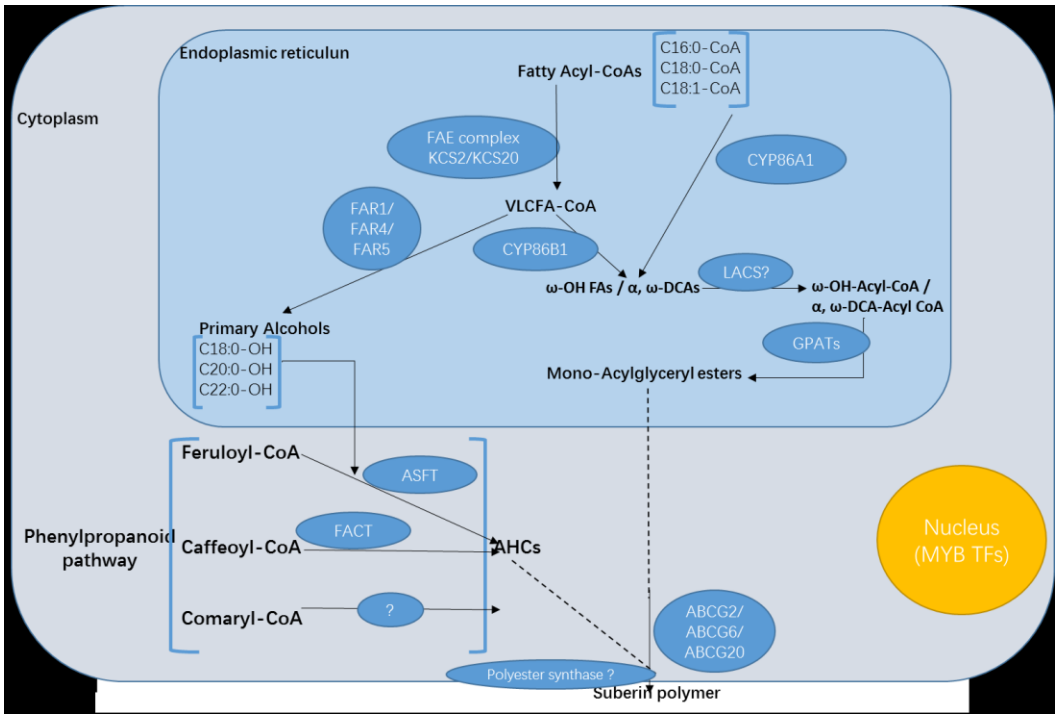


Figure 1.5. The mechanism of suberin monomer biosynthesis and suberin polymerization. Specific protein names in blue are those discovered in *Arabidopsis thaliana*. Fatty acyl elongation occurs via the fatty acid elongation (FAE) complex producing very-long-chain fatty acids (VLCFAs); acyl reduction by fatty acyl reductases (FARs) producing primary alcohols and a,ω-diols; fatty acyl oxidation by cytochrome P450 enzymes (CYPs) producing ω-hydroxy fatty acids (ω-OHs) and a,ω-dicarboxylic acids (DCAs). Esterification of ω-OHs and DCAs with glycerol-3-phosphate by glycerol 3-phosphate acyltransferases (GPATs) producing *sn*-2 monoacylglycerols. The further oxidation of ω-OHs to DCAs may be catalyzed by the same cytochrome P450s that generate the ω-OHs. Fatty acids are activated to fatty acyl-CoAs by long-chain acyl-CoA synthetases (LACSs). ATP-binding-cassette (ABC) transporters are involved in transporting suberin monomers across the plasma membrane. Polyester synthase (PS) may catalyze the esterification of *sn*-2 monoacylglycerols with other suberin monomers to produce macro-molecular polyesters. Coumaric, caffeic and ferulic acids are products from the phenylpropanoid pathway and are linked to fatty alcohols by BAHD-type acyltransferases to form alkyl hydroxycinnamates (AHCs), which are mainly present in suberin-associated waxes. ASFT, Aliphatic Suberin Feruloyl Transferase; FACT, Fatty Alcohol:Caffeoyl-CoA Caffeoyl Transferase. This figure is modified from Vishwanath *et al*., 2015.

Fatty acids are produced *de novo* in plastids and then linked with coenzymeA upon their exit to the cytosol by long-chain acyl-CoA synthetases (LACS) to generate C16 and C18 acyl-CoAs (Shockey *et al*. 2002). Although LACS enzymes have been reported to be required for activating intermediates in cutin synthesis, their roles involved in suberin biosynthesis pathway have not been investigated in detail (Vishwanath *et al.*, 2015). However, chemical analyses of *loss-of-function* mutants of the *LACS2* gene does indeed indicate additional roles in suberin formation (Li-Beisson *et al.*, 2013).

β-ketoacyl-CoA synthases (KCS), which are present as part of the endoplasmic reticulum-localized fatty acid elongase complex, are responsible for elongation of C16/C18 fatty acyl chains into very long-chain (≥C20) fatty acid derivatives (Millar and Kunst, 1997). In Arabidopsis, *DAISY/AtKCS2* was reported to be involved in suberin biosynthesis because loss-of-function *daisy/kcs2* mutants have reductions in C22 and C24 very-long-chain fatty acid derivatives in root suberin (Franke *et al*. 2008). However, the total amount of suberin in these mutants is similar to that of wild-type because of compensatory increases of shorter chain (≤C20) fatty acid derivatives (Franke *et al*. 2008). In another report, the double *kcs2 kcs20* mutant was found to have greater reductions in C22 and C24 very long-chain fatty acid derivatives in root suberin, compared with either single mutant, indicating that the two KCS enzymes function in a partially redundant manner (Lee *et al*., 2009).

A proportion of the long-chain (C16/C18) and very-long-chain (≥C20) fatty acyl-CoAs are hydroxylated to ω-hydroxy fatty acids, of which a proportion are further oxidized into α,ω-dicarboxylic acids (Agrawal and Kolattukudy 1978; Kurdyukov *et al*., 2006). This acyl oxidation process is catalyzed by members of CYP86 subfamily of cytochrome P450 monooxygenases and represents core reactions in suberin biosynthesis since ω-hydroxy fatty acids and a,ω-dicarboxylic fatty acids are the two most abundant monomers of the suberin polyester (Molina, 2010). Arabidopsis *CYP86A1/HORST* and *CYP86B1/RALPH* are involved in root suberin synthesis via production of ω-hydroxy fatty acids and α,ω-dicarboxylic fatty acids (Höfer, *et al*., 2008; Molina *et al*. 2009; Compagnon *et al.*, 2009). The *cyp86a1* mutants are characterized by a 60% decrease in total root suberin, mainly due to very significant reductions in C16, C18 ω-hydroxy fatty acids and α,ω-dicarboxylic fatty acids (Höfer *et al*., 2008). CYP86B1 is responsible for generating very-long-chain ω-hydroxy acids from C22 to C24 fatty acids. *Loss-of-function cyb86b1* mutants have almost complete loss of C22-C24 ω-hydroxy fatty acids and α,ω- dicarboxylic fatty acids, which are accompanied by increased amounts of C22-C24 fatty acids in the polymer (Compagnon *et al.*, 2009; Molina *et al.* 2009). Thus, the total suberin content remains at a level similar to wild-type.

Fatty acyl reductases (FAR) are responsible for reducing fatty acyl chains to primary fatty alcohols (Rowland and Domergue, 2012). In Arabidopsis, FAR1, FAR4

and FAR5 are involved in production of suberin-associated primary fatty alcohols (Domergue *et al*., 2010; Vishwanath *et al*., 2013). Single knock-out mutants of *FAR1, FAR4* or *FAR5* result in decreased amounts of primary fatty alcohols with chain-length specific differences both in root and seed coat suberin: 22:0-OH is mostly reduced in *far1* mutants, 20:0-OH is mostly reduced in *far4* mutants, and 18:0-OH is mostly reduced in *far5* mutants (Domergue *et al*., 2010). Consistent with these genetic results, heterologous expression of these FARs in yeast revealed that FAR1, FAR4 and FAR5 primarily produce 22:0, 20:0 and 18:0 chain length primary fatty alcohols, respectively (Domergue *et al*., 2010). Subsequently, comparison of single mutants *far1, far4,* and *far5* with the double mutants *far1 far4, far4 far5,* and *far1 far5* showed that FAR1 and FAR4 have overlapping specificities in the production of 20:0-OH and 22:0-OH, while FAR5 is almost solely responsible for 18:0-OH production (Vishwanath *et al.*, 2013). By analyzing the double mutant *far1 far4* and triple mutant *far1 far4 far5*, FAR5 was found to be also involved, to a small degree, in the production of 20:0-OH and 22:0-OH. Although these *far* mutants have decreased primary fatty alcohols to varying degrees (especially *far1 far4 far5* that had fatty alcohols reduced by 70-80%), none of these mutants are altered in other suberin constituents indicating that reduced levels of fatty alcohols does not affect the suberin polymerization process (Vishwanath *et al.*, 2013). Additionally, it was found that only about 20% of the 18:0-22:0 primary fatty alcohols

are present in suberin polymer, while the rest are founded in the non-polymeric form (i.e. as suberin-associated waxes), mostly in the form of alkyl hydroxycinnamates with alkyl caffeates predominating in Arabidopsis (Vishwanath *et al.*, 2013; Delude *et al,* 2016).

Another key enzyme family required for suberin biosynthesis are the acyl-CoA dependent glycerol 3-phosphate acyltransferases (GPATs). GPATs conduct acyl transfer reactions to produce monoacylglycerols, which function as the core suberin building blocks (Vishwanath *et al*., 2015). Arabidopsis mutants of *GPAT5* were reported to display a global 50% decrease in some monomers, including 22:0 fatty acid and 18:1 ω-hydroxy fatty acid in roots of 3-week-old seedlings (Beisson *et al*., 2007). Meanwhile, *GPAT7* is induced by wounding treatment and is responsible for producing suberin-like monomers when transiently overexpressed in leaves, suggesting its involvement in wound-induced suberin biosynthesis (Yang *et al*., 2012).

Presently, there is only one suberin-related gene identified for incorporating aromatic monomers into suberin in Arabidopsis: *ASFT/HHT* (Gou *et al*. 2009; Molina *et al*., 2009). This aliphatic suberin feruloyl transferase (ASFT/HHT) belongs to the BAHD family of acyltransferases, which catalyze the acyl transfer of feruloyl-CoA to ω-hydroxy acids and fatty alcohols to produce alkyl hydroxycinnamates (AHCs). ASFT/HHT is predicted to be a cytosol-localized enzyme, which is different from the other suberin-related enzymes described above that are localized to the endoplasmic reticulun (ER)

(Serra *et al*., 2010). *ASFT/HHT loss-of-function* mutants almost completely lack ferulate in Arabidopsis root suberin (Gou *et al*., 2009; Molina *et al.* 2009), but surprisingly this absence did not lead to obvious reductions of suberin aliphatic components or any structural alterations in the suberin lamellae (Molina *et al.* 2009).

Various suberin precursors, including monomers or partially formed oligomers, need to be transported from the ER to and across the plasma membrane, and then they are polymerized in the apoplast to form the suberin polymer. Presently, only limited knowledge of the transporters involved in suberin monomer/oligomer trafficking have been revealed. ABCG transporters are plasma membrane-localized and belong to the G-subfamily of ATP-binding cassette (ABC) transporters family. ABCG transporters were predicted to be involved in suberin monomer/oligomer transport since they are responsible for export in the deposition of other plant extracellular lipid polymers, such as cuticle (cutin and waxes) and sporopollenin (Pighin *et al*., 2004; Choi *et al*., 2011). In Arabidopsis, an *abcg2 abcg6 abcg20* triple mutant was characterized and shown to have alterations in the structure, composition and properties of root and seed coat suberin (Yadav *et al.,* 2014). In other species, such as rice and potatoes, ABC transporters OsABCG5 and StABCG1 are also involved in the suberization of the hypodermis of rice roots (Shiono *et al*., 2014) and the periderm of potato tubers (Landgraf *et al*., 2014), respectively. Additionally, it is possible that the larger and/or branched oligomers would

require a different transport mechanism involving exocytotic vesicles or oleophilic droplets as described in rapidly expanded rice internodes (Hoffmann-Benning *et al.*, 1994). However, before ABC transporters are involved in lipid export, a secretory vesicle trafficking system between the endoplasmic reticulum (where monomers/oligomers are produced) and the plasma membrane also contributes to extracellular lipid deposition. For example, in the transport of cuticular wax, significant decreases of stem wax deposition were observed in vesicle-trafficking mutants *gnl1-1* and *ech*. This indicates a role for GNL1- and ECH-mediated vesicle trafficking in the export of cuticular wax (McFarlane *et al*., 2014). Vesicle trafficking may similarly be involved in suberin deposition.

Polyester synthases responsible for lipid polymer assembly have been identified for cutin, but no candidates have yet been identified for suberin polymerization. In tomatoes, the extracellular protein CD1 of the GDSL-motif lipase/hydrolase family catalyzes transesterification of *sn*-2-(10,16-dihydroxyhexadecanoyl)-monoacylglycerol cutin precursors *in vitro* (Yeats *et al*., 2012; Girard *et al*., 2012). Recently, a homolog from Arabidopsis was found and characterized *in vitro*, but it only polymerized linear chains and not branched-chain products thought to be characteristic of the suberin polymer (Yeats *et al*., 2014). Another cell wall-localized protein called BODYGUARD is also involved in cutin polymerization. *Bodyguard* loss-of-function mutants display a series of phenotypes indicating disrupted cuticle formation (Kurdyukov *et al*., 2006).

In Arabidopsis, suberin-associated waxes (also known as root waxes) are found in root endodermis and mature root periderms and are mostly composed of alkyl hydroxycinnamates (AHCs), representing about 90% of the total waxes in Arabidopsis - among them, 18:0-22:0 alkyl caffeates are the most abundant components (Kosma *et al.*, 2015; Delude *et al*., 2016). An acyl transferase (FACT) is responsible for the synthesis of unpolymerized alkyl caffeates in Arabidopsis root (Kosma *et al*., 2012). Meanwhile, it has been suggested that the synthesis of both suberin polymer and the non-covalently linked waxes from suberized layers have common biosynthetic origins. For example, the fatty acyl-CoAs elongases KCS2 and KCS20 are necessary for the formation of the very-long-chain aliphatics present in the suberin polymer as well as those of suberin-associated waxes (Delude *et al*., 2016). Similarly, FARs are responsible for the synthesis of 18:0-22:0 primary fatty alcohols present in both suberin polymer and the counterparts found in suberin-associated waxes (Kosma *et al*., 2012; Vishwanath *et al*., 2013).

1.6 MYB transcription factors in plants

The *v-myb* myeloblastosis viral oncogene homolog (MYB) family of transcription factors (TFs) is widely distributed in land plants and comprises one the largest group of TFs in plants. Its members play important roles in controlling growth and development, stress responses, and production of specialized (secondary) metabolites

(Dubos *et al*., 2010). The MYB-TFs are characterized by a highly conserved DNA-binding domain: the MYB domain, which is present as one to four imperfect repeats (R), each of which includes a sequence of about 52 amino acids (Ogata *et al*., 1996).

According to the number and placement of the repeats, MYB proteins can be classified into four groups: 1R (R1/2 or R3-MYB), 2R (R2R3-MYB), 3R (R1R2R3-MYB), and 4R (4 R1/R2-like repeats)-MYB subfamilies (Dubos *et al*., 2010; Liu *et al*., 2015) (Figure 5). The basic structures and functions of each plant MYB-TF group are described as follows. 1R-MYB consists of proteins with a single R3-type or a partial R1/R2 MYB repeat. 1R-MYB proteins participate in morphogenesis, control of specialized metabolism, circadian clock control, response to phosphate starvation, and flower and fruit development (Feller *et al*., 2011). 2R-MYB is the predominant MYB class in plants (Stracke *et al*., 2001; Matus *et al*., 2008； Wilkins *et al*., 2008; Du *et al*., 2012). 2R-MYB with a R2R3-type repeat has a modular structure, consisting of a DNA-binding domain (the MYB repeats) at the N terminus and a highly variable activation or repression domain at the C terminus. Based on amino acid sequence conservation in the MYB domain and the C terminal domains, R2R3-MYB proteins are divided into 28 subgroups (Stracke *et al*., 2001; Dubos *et al*., 2010). These R2R3-MYB proteins are involved in central and specialized metabolism, determination of cell fate and identity, hormone signal transduction, regulation of development, and response to

biotic and abiotic stresses (Dubos *et al*., 2010). 3R-MYBs, with a R1R2R3-type repeat, have been identified in most eukaryotic genomes, including animals. These proteins are mainly associated with control of the cell cycle (Ito 2005; Haga *et al*., 2007). 4R-MYB is the smallest class group and very little is known about these proteins in plants (Dubos *et al*., 2010). In Arabidopsis, the MYB gene family is represented by only five 3R-MYB genes, compared with 190 R2R3-MYB genes (Stracke *et al*., 2001; Yanhui *et al*., 2006).

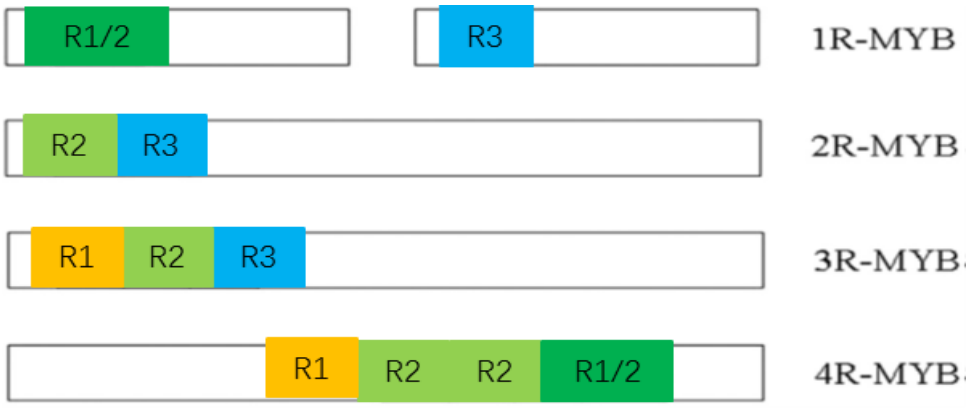


Figure 1.6. Schematic representations of plant MYB transcription factor groups. Figure is modified from Liu *et al*., 2015.

The MYB repeats are involved in direct DNA binding. Both the R2 and R3-MYB repeats are necessary for direct DNA binding, but neither R2 nor R3 can alone bind DNA specifically (Ogata *et al*., 1994). Structural studies showed that the R2 and R3

recognition helices contact directly with each other before sequence-specific binding to target DNA (Tahirov *et al*., 2001; Tahirov *et al*., 2002). Thus, both R2 and R3 repeats bind cooperatively to their cognate DNA target sequences. Meanwhile, 1R-MYB proteins, which contain only one repeat, cannot directly bind to DNA but need to first bind to other transcription factors (Koering *et al*., 2000; Hwang *et al*., 2001).

The three MYB subfamilies including 1R-, 2R- and 3R-MYB groups each preferentially bind to different DNA sequences. 1R-MYB members bind predominantly to the telomeric sequence TTAGGG (Martin and Paz-Ares, 1997). The R2R3-MYB members share relatively high similarity in terms of amino acid sequence in their recognition helices, and have similar DNA-recognition patterns (Romero *et al*., 1998). Based on amino acid sequence similarities, R2R3-MYB family members are classified into three phylogenetic clades (group A, B, and C) and each of the clades has been analyzed for DNA-binding specificities (Romero *et al*. 1998). It is known that members of group A bind MBS (MYB Binding Site) type I sequences (C(A/C/G/T)GTT(A/G)), members of group B bind equally to both MBS type I and type II sequences (G(G/T)T(A/T)GTT(A/G)), and most members of group C bind MBS type IIG sequences ((C/T)ACC(A/T)A(A/C)C). 3R-MYB factors in tobacco were found to bind to the sequence AACGG (Ito *et al*. 1998). It is still possible, however, that proteins from each of the three subfamilies do not follow the DNA binding patterns described above (Li and

Parish 1995; Lu *et al*.. 2002). Therefore, it is necessary to conduct research to identify the MYB-TFs DNA binding sites for individual MYB proteins because it is difficult to predict MYB DNA-binding sites based solely on amino acid sequences of the R repeats (Prouse and Campbell, 2012).

An increasing number of MYB proteins have had their direct gene targets elucidated (Dubos *et al*., 2010). According to information currently provided by the platform of AGRIS and AtRegNet (Palaniswamy *et al*. 2006), a total of 552 direct target genes of MYB proteins have been revealed. These MYB factors include 3R-MYBs (*MYB3R4* and *MYB3R1*) and five R2R3-MYBs (*AtMYB123, AtMYB2, AtMYB66, AtMYB0* and *AtMYB33*). Among these MYB factors, *At*MYB0 and *At*MYB66 directly target genes encoding other transcription factors (Koshino-Kimura *et al*. 2005), whereas other MYB factors target non-transcription factor genes (Abe *et al*., 2003; Baudry *et al*., 2004; Haga *et al*., 2007).

1.7 Transcription factors regulating suberin biosynthesis

Suberin deposition is both spatially and temporally restricted, as well as inducible by stresses. This is controlled to a large degree at the level of transcription, which is supported by expression studies of suberin biosynthetic genes (Ranathunge *et al*., 2011; Vishwanath *et al.*, 2015).

Presently, only a few transcription factors (TFs) have been reported or predicted to regulate suberin biosynthesis. The transcript levels of genes encoding TFs of the WRKY, NAM (No-Apical-Meristem)-like, AS1-interacting KH protein, and SQUAMOSA promoter-binding protein-like (SPL) families are strongly up-regulated in suberized cork (Soler *et al.*, 2007). Thus, these TFs may be involved in suberin biosynthesis in cork (phellem tissue). *At*ERF38, which belongs to AP2/ERF-type (APETALA2/ ethylene-response-factor) TF family in *Arabidopsis thaliana*, has been suggested to function in suberin production during plant development (Lasserre *et al*., 2008). Transgenic lines harbouring a *At*ERF38 promoter::GUS fusion revealed *At*ERF38's strong expression specifically in suberized tissues including the outer integument of mature seeds, endodermis of roots in the primary developmental stage, and sclerified tissues of mature inflorescence stems. However, this gene co-expression pattern does not prove that it is regulating suberin biosynthetic genes and further experiments are required to test its role. Also, analysis of promoter sequences of upregulated genes in suberin biosynthesis are enriched in putative *cis*-elements that are associated with the transcription factors containing WRKY or AP2 domains (Shiono *et al*., 2014). However, these studies did not provide conclusive evidence for any TFs directly controlling transcriptional regulation of suberin biosynthetic genes.

1.8 MYB Transcription factors regulating suberin biosynthesis

Cork cells are highly suberized during the secondary growth stage. QsMYB1 is one of the candidate TFs important for cork formation in cork oak tree (*Quercus suber*). It was found that *QsMYB1* transcripts were upregulated in suberized cork tissue with alternative splicing (AS) variants, showing a high expression of two different transcripts of *QsMYB1.1* and *QsMYB1.2* (Almeida *et al*., 2013). However, no evidence has yet been provided to show that these MYB TFs directly bind the promoters of suberin biosynthetic genes.

A study from the Rowland Lab showed that *At*MYB41 strongly induces suberin production when it was stably overexpressed in leaves of *Arabidopsis thaliana* or transiently overexpressed in leaves of *Nicotiana benthamiana* (Kosma *et al*., 2014). The concomitant significant increases in transcript levels of all known suberin biosynthetic genes further indicated that MYB41 is involved in regulating suberin production. Additionally, transgenic *MYB41* promoter: GUS lines subjected to unstressed conditions versus abscisic acid (a stress hormone) or high NaCl treatments showed that *MYB41* is active in root endodermis under stressful condition but not during normal growth.

In *Malus*×*domestica*, MYB93 was reported to positively regulate suberin deposition. RNA-seq analysis shown that transient overexpression of *MdMYB93* up-regulated a core cluster of predicted suberin biosynthetic genes and also induced several

lignin- and cutin-related genes. Meanwhile, a massive accumulation of suberin polymer as well as free fatty acids and phenylpropanoids were observed (Legay *et al*., 2016). Although there is no direct evidence showing that the MYB93 ortholog from Arabidopsis (*At*MYB93) regulates suberin deposition (Gibbs *et al.*, 2014), *Md*MYB93 has high amino acid similarities with *At*MYB93 in the N-terminal region DNA binding domains (Legay *et al*., 2016). This suggests that *At*MYB93 has similar regulatory functions in suberin biosynthesis.

Recently, *At*MYB107 was shown to be essential for positive suberin regulation in the seed integument layer (Gou *et al*., 2017). Featured with low permeability and low germination rates under stress, *myb107* loss-of-function mutants display significant (50%-60%) decreases in seed coat suberin. According to chromatin immunopreciptation-qPCR results, MYB107 was shown to directly interact with the promoters of the suberin related genes *FAR1, GPAT5, HHT,* and *FACT*.

Through a broad comparison study of suberin deposition in Arabidopsis seeds, several organs of tomato (*Solanum lycopersicum*) including suberized fruit skin, russeted apple (*Malus x domestica*) fruit surfaces, grapevine (*Vitis vinifera*), potato (*Solanum tuberosum*), and the roots of rice (*Oryza sativ*) under waterlogging conditions (Lashbrooke *et al*., 2016), predicted MYB107 and MYB9 orthologs were found to be co-expressed with suberin biosynthetic genes in these plant species. Further, in Arabidopsis

T-DNA *loss-of-function* lines *MYB9* and *MYB107*, suberin biosynthetic genes were down-regulated while suberin accumulation was also decreased in seed coat. Additionally, it is likely that *AtMYB107* and *AtMYB9* influence each other's expression because there is an increase in *MYB107* transcripts in *myb9* mutants. This probably partially compensates phenotypically for the downregulation of MYB9 (Lashbrooke *et al*., 2016).

1.9 Project rationale and objectives

The biosynthesis of suberin has attracted much interest in the past 10 years, with major advancements achieved using the model plant *Arabidopsis thaliana* and the crop *Solanum tuberosum* (potato). Most research has been focused on the endoplasmic reticulum-localized enzymes involved in suberin monomer formation and the plasma membrane-localized transporters involved in excretion of suberin monomers or oligomers to the cell wall (Vishwanath *et al*., 2015). The genes encoding these enzymes are spatially and temporally controlled during normal growth and development as well as induced by various abiotic stresses (Ranathunge *et al*., 2011; Vishwanath *et al*., 2015). However, the molecular mechanisms involved in suberin-related gene regulation are not well understood. Importantly, the identities of transcription factors directly or indirectly controlling the transcriptional regulation of suberin biosynthetic or transporter genes need to be identified (Vishwanath *et al*., 2015). The controlled deposition of suberin in roots is

of particular interest given the key role of root suberin in controlling water and nutrient uptake, and its role in protection against uptake of toxic substances and root pathogens (Franke and Schreiber, 2007; Schreiber, 2010; Geldner, 2013; Barberon and Geldner, 2014). Presently, only Arabidopsis MYB41 transcription factor (TF) has reported to be an activator of suberin biosynthesis in roots, but it functions only under certain stress conditions, such as high salt (Kosma *et al*., 2014).

Recent research in the Rowland lab (Murmu *et al*., manuscript in preparation) indicated that the highly related Arabidopsis MYB53, MYB92, and MYB93 transcription factors together function in regulating suberin biosynthesis during normal root development (unstressed conditions). This is based on the following evidence so far: (1) transient overexpression of MYB53, MYB92 or MYB93, which are closely related MYB-type transcription factors, in leaves of *Nicotiana benthamiana* resulted in ectopic suberin deposition in leaf cell walls; (2) use of transgenic Arabidopsis promoter::reporter gene fusion lines revealed that *MYB53*, *MYB92* and *MYB93* are expressed in young root endodermis where suberin is normally deposited and including under non-stress conditions; (3) *loss-of-function* single and double mutants of *MYB92* and *MYB93* exhibited a reduction in root suberin, specifically in root endodermis. However, the role of MYB53 in root suberin production was unclear due to the unavailability of *loss-of-function* T-DNA insertion mutants of *MYB53* at that time. My hypothesis is that the

R2R3-MYB transcription factor MYB53 from *Arabidopsis thaliana* functions with MYB92 and MYB93 to positively regulate suberin deposition during normal plant root development. To test this, I took advantage of some recently reported *MYB53* T-DNA insertion lines and by using a stable Arabidopsis transgenic line that allows for the rapid overexpression of MYB53 upon application of the steroid β-estradiol.

Chapter 2: Materials and Methods

2.1 Plant materials and growth conditions

Arabidopsis thaliana plants were the Columbia (Col-0) ecotype background. The T-DNA insertion lines SALK_076713 (*myb53-1*), CS853878 (*myb53-2*), SM_3_41690 (*myb92-1*) and SALK_131752 (*myb93-1*) were obtained from the Arabidopsis Biological Resource Centre (http://www.arabidopsis.org/). The double mutants *myb53-1 myb92-1, myb53-1 myb93-1, myb53-2 myb92-1,* and *myb53-2 myb93-1* were created by crossing single mutants *myb53-1* or *myb53-2* with *myb92-1* or *myb93-1*. The double mutant *myb92-1 myb93-1* was generated by crossing single mutants *myb92-1* and *myb93-1*. The triple mutants *myb53-1 myb92-1 myb93-1* and *myb53-2 myb92-1 myb93-1* were created by crossing *myb92-1 myb93-1* with *myb53-1* and *myb53-2*, respectively. Plants homozygous for T-DNA insertions in the *MYB53*, *MYB92*, and *MYB93* genes in the single, double or triple mutants were identified in the F2 population by PCR genotyping. The primers (gene-specific and T-DNA-specific) used for PCR genotyping are listed in Appendix A. The TRANSPLANTA-559 (TPT-559) line for inducible overexpression of *MYB53* was obtained from the TRANSPLANTA consortium collection (http://bioinfogp.cnb.csic.es/transplanta_dev), which is described in Coego *et al*. (2014).

All *A. thaliana* seeds (wild-type, mutants, or TRANSPLANTA line) were surface sterilized by immersing them 70% ethanol for about 2 min, followed by immersion in

20% bleach plus 0.1% Tween20 (Cat #. TWN510.500) for about 5 min, and then the sterilized seeds were washed 4 times with sterile distilled water. The sterilized seeds were stratified for 3-4 days at 4℃. Plants were either grown on AT media [5 mM KNO_3, 2.5 mM KH_2PO_4, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 50 μM $Fe_4(EDTA)_3$ and 1 mM micronutrients] or in sterilized potting soil (Pro-mix BX-General Purpose growing medium, Cat #. 10380RG) fertilized with 1 g/L fertilizer [Plant-Prod, 20(N)-20(P)-20(K) Classic, Cat #. 10529] plus 0.0625 g/L micronutrients (Plant-Prod chelated micronutrient mix). All plants were grown in an environmental growth chamber at 22-23℃ with 30–60% humidity, a 16h/8h light/dark cycle, and a light intensity of 149-152 μmol• m^{-2}•s^{-1}.

2.2 Chemical inducement of MYB53 in TPT-559 overexpression line

The steroid β-estradiol (E–8875, Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) and added to AT media at a final concentration of 20 μM. About 20-25 seeds were grown on narrow strips (5 mm width × 100 mm length) of sterilized filter paper (Fisher Scientific, Cat #. 09790E) and placed on the surfaces of solidified AT media in square petri plates (Fisher Scientific, Cat #. FB0875711A). All the square plates were kept in a vertical position in the same environmental growth chamber and under the same conditions as described above. The rows of 10-day-old TPT-559 seedlings were transferred using sterilized tweezers from normal AT media to β-estradiol-containing AT

media for a time-course of growth (1 day, 4 days, 7 days or 10 days). The negative controls were simultaneously transferred in the same way to AT media containing the same amount of DMSO solvent only.

2.3 RNA preparation and cDNA synthesis

To analyze the *MYB53, MYB92*, and *MYB93* transcript levels in the different lines, plant tissues were harvested from 2-weeks-old roots (in the case of T-DNA insertion mutants and corresponding wild-type ecotype) or from 10-days-old roots or leaves (in the case of TPT-559 inducible overexpression line, with or without β-estradiol). Plant roots and leaves were separately wrapped in aluminum foil and immediately submerged in liquid nitrogen. For long-term storage, tissues were stored at -80°C. Microcentrifuge tubes (1.5 mL) and spatulas were pre-chilled in liquid nitrogen and remained chilled throughout the procedure by using a rack submerged in liquid nitrogen. Frozen plant tissue was placed in a liquid nitrogen-chilled mortar and ground into fine powders using a chilled pestle. The powders were quickly transferred into chilled micro-centrifuge tubes using chilled spatulas. The NORGEN plant/fungi total RNA purification kit (Cat #. 25800, NORGEN BIOTEK CORP., https://norgenbiotek.com/) was used according to the manufacturer's protocol. Genomic DNA remaining in the extracted total RNA was degraded using the Ambion TURBO

DNA-free kit (Cat #. AM1907, Thermo Fisher Scientific, https://www.thermofisher.com/). The reaction system was set up in a total volume of 30 µl, including 25 µl total RNA, 3 µl 10×DNase buffer (supplied in the kit), 1 µl DNase (supplied in the kit), and 1 µl RNase OUT Recombinant Ribonuclease Inhibitor (Cat #. 10777019, Thermo Fisher Scientific, https://www.thermofisher.com/). The integrity and concentration of purified RNA was verified by agarose gel electrophoresis and using a Nanodrop spectrophotometer. One µg of DNase-treated purified RNA was used as template for first-strand cDNA synthesis using the iScript cDNA Synthesis Kit (Cat #. 1708891, Bio-Rad, http://www.bio-rad.com/) according to the manufacturer's instructions. The cDNA product was diluted 10 times with TE buffer [10mM Tris- HCl (pH 7.5) and 1 mM EDTA (pH 8.0)], distributed into many aliquots, and stored at -80°C for long-term storage.

2.4 Semi-quantitative RT-PCR analysis of transcript levels

For semi-quantitative RT-PCR, the reference gene *GAPDH* (At1g13440) was used as an endogenous control. The reactions were conducted at 58℃ annealing temperature and the cycle numbers were 26 for *GAPDH* and 30 for the *MYB53/92/93* genes. The primers are listed in Appendix B Table 8. The 10-times diluted cDNA was used as template in each 20-µl PCR system. The PCR products were then separated on a

1% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized using an AlphaImager 2200 (Alpha Innotech, Santa Clara CA).

2.5 Quantitative PCR analysis of transcript levels

Quantitative PCR was performed on a Bio-Rad CFX Connect Real-Time PCR System using SSoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (Cat #. 1725017, Bio-Rad, http://www.bio-rad.com/). Each qPCR reaction contained 5 μl of 2X SYBR Green Supermix, 0.5 μl primer mix (250 nM as the final concentration for both forward and reverse primer), 2 μl of 10x diluted cDNA, and 2.5 μl autoclaved nano-pure water. The primers are listed in Appendix B, Table S9. For each pair of primers, the amplification efficiency of all the genes of interest and reference genes were found to be between 91% -112% (Table 2.1). The PCR thermal profile was composed of 1 cycle at 98℃ for 3 min, followed by 40 cycles at 98℃ for 15 sec, and then 60℃ for 30 sec. For each run, melt curve analysis was conducted at the end of the amplification to make sure that no primer dimers or non-specific amplicons were formed. This thermal profile was conducted between 65-95℃ at 0.5℃ increments with 5 sec per step.

Table 2.1 The PCR primer efficiency for suberin biosynthetic genes and reference genes

Arabidopsis Gene ID	Annotation	PCR Efficiency (×100%)
At5g58860	*CYP86A1*	93
At5g23190	*CYP86B1*	97
At3g11430	*GPAT5*	91
At5g41040	*ASFT*	105
At5g22500	*FAR1*	103
At3g44540	*FAR4*	101
At3g44550	*FAR5*	112
At1g13440	*GAPDH*	98
At1g13320	*PP2A*	100

Transcripts were quantified as relative abundance using the comparative Ct method "delta-delta Ct: $2^{-\Delta\Delta Ct}$". The reference genes *GAPDH* (At1g13440) and *PP2A* (At1g13320) (Czechowski *et al*., 2005) were used as constitutive controls in each sample to normalize the differences of total RNA content across all samples. Three biological replications were conducted for each condition, with two technical replicates for each sample.

2.6 Lipid polyester (suberin / cutin) analysis using gas chromatography

Roots from 2-weeks-old seedlings were harvested for quantitative characterization of lipid polyester composition (suberin) in the T-DNA loss-of-function mutant lines compared to wild-type. For TPT- 559 (*MYB53*) inducible overexpression line, with or

without steroid treatment, roots and leaves from 11 day-, 14 day-, 17 day- and 20 day-old seedlings (i.e. 10-day-old seedlings were exposed to β-estradiol treatment) were harvested for characterization of lipid polyesters (suberin in roots and cutin + suberin in leaves). At least three replicates of each sample were prepared for suberin / cutin chemical analyses, and approximately 180 pooled seedlings were collected for each T-DNA loss-of-function mutant biological replicate and approximately 100 pooled seedlings for each overexpression biological replicate.

Tissue delipidation (removal of non-polymerized lipids) and base-catalyzed de-polymerization of lipid polyesters were carried out prior to suberin analysis by gas chromatography. To avoid any carry over of external lipid contamination, all glassware was cleaned three times with chloroform and all glass tube caps were rinsed three times with 50% methanol. For delipidation, all samples of roots and leaves tissue were rapidly collected after being chopped into pieces and immersed in hot isopropanol (pre-heated in an 85°C oven) for 15 min at 85°C. After cooling to room temperature, broken tissue was further ground up using a small hand-held polytron that had also been rinsed three times with chloroform. Later, soluble lipids were removed from the ground samples (delipidation) by incubating tissues with a series of mixed solvents of increasing polarity: 2:1 (v/v) chloroform : methanol, 1:1 (v/v) chloroform : methanol, (1:2) (v/v) chloroform : methanol, and 100% methanol, successively (chloroform, HPLC grade, Cat #. CA71006-

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

After delipidation, all samples were dried in a fume hood at room temperature for several days and then transferred to a sealed desiccator with fully dried drierite (calcium sulfate) for at least a few days. The resulting dried residues were weighed. Ten μg of each internal standard, 17:0 methyl ester and omega-pentadecanolactone (OPL), were added to each sample replicate. The lipid polyesters were depolymerized by base-catalyzed trans-esterification at 60°C for 2 h by adding 0.9 ml methanol, 0.225 ml methyl acetate, and 0.375 ml sodium methoxide. Samples were vortexed thoroughly every 30 min during the reaction. After cooling down, one ml of glacial acetic acid was added to stop the reaction to adjust the pH between 4 and 5. Then, 2.5 ml of dichloromethane (methylene chloride) and 2 mL of 0.5 M NaCl was added to each sample to extract the depolymerized monomeric constituents. The mixtures were vortexed thoroughly and centrifuged for 5 min at 805×g. The compounds were obtained in the organic phase, which was then washed three times with 1 ml of 0.5 M NaCl, extracting into the organic phase each time. Anhydrous sodium sulfate (around one-half volume of the organic phase) was added to remove residual water from the organic phase. Samples were

centrifuged at 805 × g the next day and the anhydrous organic phase transferred to clean tubes. Extracts were dried under a gentle stream of nitrogen at 37°C for about 15 min. The free hydroxyl groups were then acetylated (derivatized) using 0.1 ml acetic anhydride + 0.1 ml pyridine at 60°C for 1 h. The samples were again evaporated under a gentle stream of nitrogen at 37°C. Dried samples were then re-dissolved in 0.1 ml hexane for analysis by gas chromatography.

A Varian 450-GC gas chromatograph equipped with a FID (Flame Ionization Detector) was used for analysis. One μl of each sample was injected using a glass 10 μl Hamilton syringe fitted onto an autosampler. The samples were injected into a split/splitless injector at 300°C using a 1:20 split ratio. Individual monomers were separated on an Agilent J&W GC capillary column (HP-5MS, 30 m length, 0.250 mm diameter, 0.25 μm film thickness). The oven temperature program was: initial temperature of 140°C for 3 minutes, $10°C \cdot min^{-1}$ ramping up to 310°C, and then held at 310°C for 17 minutes. The monomers were detected by FID set at 325°C. The total run time for each sample was 30 minutes. Helium was used as carrier gas at a constant flow of 2.0 ml/min, nitrogen was used as make-up gas, and air and hydrogen were used as combustion gas. Peak identification was done by comparison with retention time patterns of previous extracts that had been characterized by gas chromatography – mass spectrometry (GC-MS).

2.7 Quantification of suberin monomer amount using flame ionization detection

The FID response is proportional to the carbon atom number and the degree of unsaturation of the analytes. Therefore, to calculate these variant FID responses to individual components, correction factors are used to amend the peak areas of the signal produced by individual suberin monomers and then detected by FID (Mossoba, *et al*., 2014). The FID correction factor for each monomer is determined by the number of carbon atoms in the hydrocarbon molecule, which is the trimethylsylil methyl derivatized monomer:

$$\text{FID correction factor} = \frac{\text{the number}_{(\text{carbon atoms forming C-H bonds})} \times 12}{\text{molecular weight}_{(\text{trimethylsylil methyl derivatized monomer})}}.$$

$$\text{Therefore, the corrected peak area} = \frac{\text{peak area}}{\text{FID correction factor}}.$$

The corrected peak areas of the suberin monomers were then compared to the corrected peak areas of 17:0 methyl ester and omega-pentadecanolactone (OPL) as the two internal standards (IS). For both of these two internal standards, 10 μg was added to each sample to determine the amount of each suberin monomer, which was represented as the

$m_{(\text{suberin monomers})}$:

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

Since all monomers were acetylated, $m_{(\text{subeirn monomer})} = n_{(\text{acetylated suberin monomer})} \times MW_{(+\text{acetate})}$.

Thus,

$$n_{(\text{acetylated subeirn monomer})} = \frac{\text{corrected peak area}_{(IS)} \times 10\ \mu g}{\text{corrected peak area}_{(\text{suberin monomer})} \times \text{molecular weight}_{(+\text{acetate})}}$$

The $n_{(\text{acetylated suberin monomer})}$ was used to calculate the actual amount of suberin monomers as the unacetylated suberin monomers in the original polymer:

$$m_{(\text{unacetylated suberin monomer})} = n_{(\text{acetylated suberin monomer})} \times \text{molecular weight}_{(-\text{acetate})}.$$

Chapter 3: Results

3.1 Ectopic production of suberin in roots and leaves by steroid-induced overexpression of Arabidopsis *MYB53*

A β-estradiol inducible overexpression system was used to examine whether *MYB53* overexpression leads to changes in suberin production (Figure 3.1). Specifically, *MYB53* overexpression was induced by using the Arabidopsis TRANSPLANTA-559 (TPT-559) overexpression line (Coego *et al.,* 2014). This transgenic line contains two transcriptional units jointly functioning for specifically triggering the overexpression of *MYB53* upon application of the steroid β-estradiol. In the first transcription unit, a synthetic constitutive promoter G10-90 (Ishige *et al.*, 1999) drives the expression of XVE activator (Zuo *et al.*, 2000), which is a fusion of the LexA DNA binding domain (X), VP16 transactivation domain (V), and the hER regulatory domain of the human estrogen receptor (E). Upon binding β-estradiol, this chimeric XVE factor enters the nucleus and

stimulates the transcription of a downstream target gene (*MYB53* in this case), which is controlled by a second promoter consisting of eight copies of the LexA operator fused with the -46 35S minimal promoter (Benfey *et al.*, 1990).

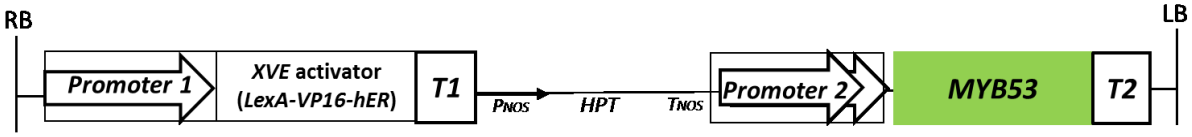


Figure 3.1. Schematic representation of the chemical inducible system for overexpressing Arabidopsis MYB53 (At5g65230).
The schematic was modified from Zuo *et al*., 2000; Coego *et al*., 2014. Promoter 1 is a synthetic promoter (Ishige et al., 1999) controlling the expression of chimeric XVE trans-activator. The encoded XVE trans-activator protein sequence contains the DNA binding domain of LexA, the transcription activation domain of VP16, and the regulatory domain of the human estrogen receptor (hER). T1 is the rbcS E9 terminator sequence used for the XVE activator transcription unit. Promoter 2 is composed of 8 copies of the LexA Operator sequence and the -46 35S minimal promoter. MYB53 is the coding sequence of AtMYB53 transcription factor. T2 is the rbcsS 3A terminator sequence used for the MYB53 transcription unit. Between these two transcription units is the hygromycin phosphotransferase II coding sequence (HPT), which confers hygromycin resistance in plants. This selective marker is under the control of the nopaline synthase promoter (Pnos) and the nopaline synthase terminator sequence (Tnos).

Ten-day-old seedlings were treated with 20 μM β-estradiol and then roots and leaves were harvested for lipid polyester analysis 1 day, 4 days, 7 days and 10 days after

treatment. The seedlings were grown vertically in square tissue culture plates for the convenience of rapidly transferring seedlings to mock- and hormone-treated medium while maintaining the integrity of the roots (i.e. minimize stress). After 16 hours of steroid treatment, the *MYB53* transcript was induced in both roots and leaves (Figures 3.2A and 3.3A). In roots (Figure 3.2B), there was no significant difference in the amount of suberin one day after incubating with hormone (20.73 μg/mg suberin) in comparison to mock-treated (DMSO only) control (17.73 μg/mg suberin). However, suberin total content increased significantly and reached 95.36 μg/mg 4 days after overexpressing *MYB53* compared with 20.57 μg/mg suberin in roots of the mock-treated control. After 7 and 10 days, the suberin amount gradually decreased to 80.44 μg/mg and 48.83 μg/mg, respectively, in roots of hormone-treated plants, but it was still significantly higher than the mock control at each of these time points (18.68 μg/mg and 15.09 μg/mg, respectively). Meanwhile, *MYB53* overexpression also significantly increased leaf lipid polyester content (cutin plus suberin, as these will be depolymerized together) after 4 days, 7 days and 10 days of hormone treatment compared to mock control, but there was no obvious increase after 1 day of treatment (Figure 3.3B). The leaf lipid polyester significantly increased to 7.16 μg/mg, 9.04 μg/mg and 6.30 μg/mg, respectively, after 4 days, 7 days and 10 days of treatment, compared to the mock control at each of these time points (4.02 μg/mg, 5.00 μg/mg and 3.33 μg/mg, respectively).

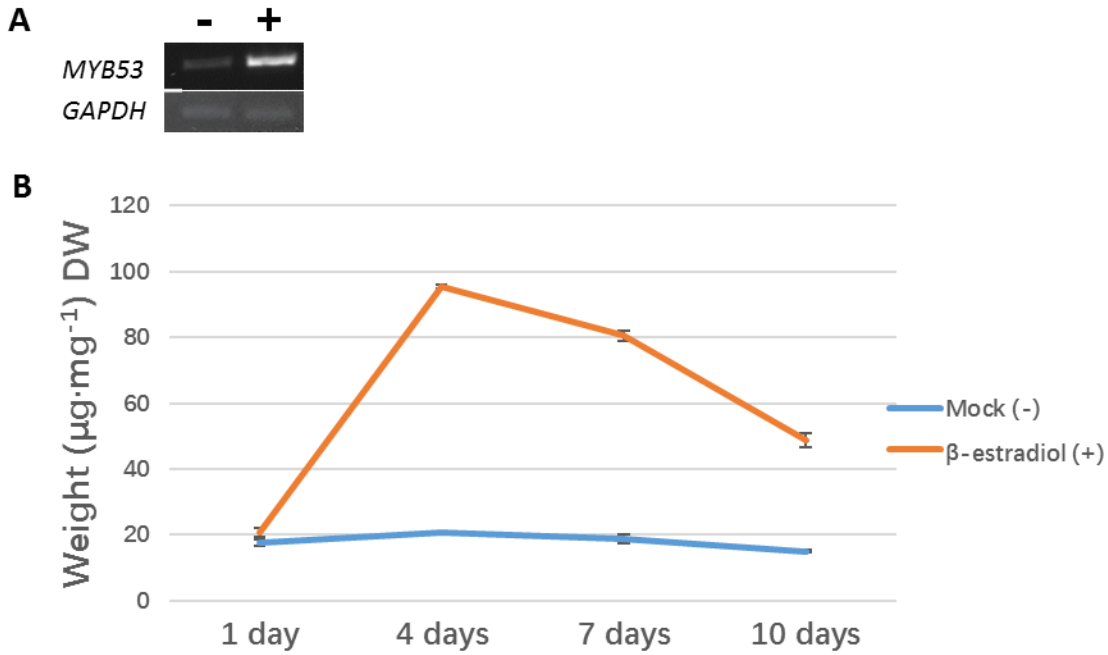


Figure 3.2. Suberin analysis in roots of the *MYB53* inducible overexpression line over a time-course.

(A) RT-PCR analysis of *MYB53* transcript levels in roots of the *MYB53* overexpression line treated with β-estradiol (+) or DMSO-alone mock control (-). *GAPDH* (At1g13440) was used as the constitutively expressed reference gene; the RNA sample was harvested 16h after applying DMSO alone and DMSO with β-estradiol for the mock control (-) and treated groups, respectively. (B) Total suberin content in roots of the *MYB53* overexpression line treated with β-estradiol (+) or DMSO-alone mock control (-). The β-estradiol and the DMSO were applied to 10-day-old seedlings and then grown for 1 day, 4 days, 7 days or 10 days. The total suberin amount is expressed as $\mu g \cdot mg^{-1}$ dry weight root tissue. Error bars = SE, n=3 replicates. The asterisks represent the statistical significance ("*" represents $p<0.05$, "**" represents $p<0.01$, "***" represents $p<0.001$, least-significant difference (LSD) comparison).

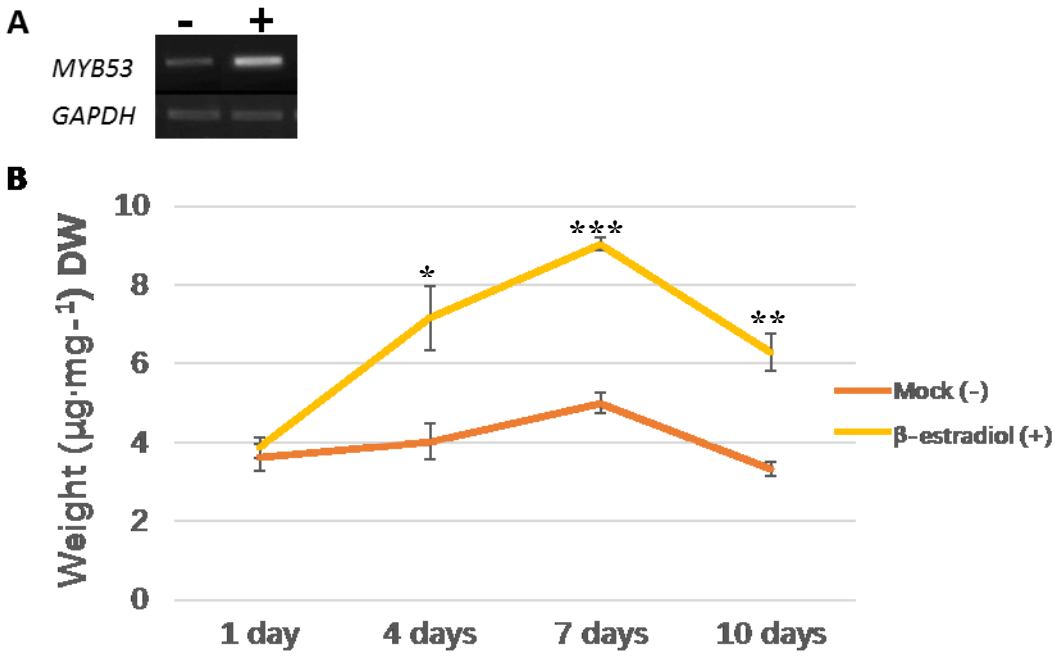


Figure 3.3. Total lipid polyester (cutin and suberin) analysis in leaves of the *MYB53* inducible overexpression line over a time-course.

(A) RT-PCR analysis of *MYB53* transcript levels in leaves of the *MYB53* overexpression line treated with β-estradiol (+) or DMSO-alone mock control (-). *GAPDH* (At1g13440) was used as the constitutively expressed reference gene; the RNA sample was harvested 16h after applying DMSO alone and DMSO with β-estradiol for the mock control (-) and treated groups, respectively. (B) Total lipid polyester (cutin + suberin) content in leaves of the *MYB53* overexpression line treated with β-estradiol (+) or DMSO-alone mock control (-). The β-estradiol and the DMSO were applied to 10-day-old seedlings and then grown for 1 day, 4 days, 7 days or 10 days. The total suberin amount is expressed as $\mu g \cdot mg^{-1}$ dry weight root tissue. Error bars = SE, n=3 replicates. The asterisks represent the statistical significance ("*" represents $p<0.05$, "**" represents $p<0.01$, "***" represents $p<0.001$, LSD comparison).

We then examined in detail the changes in chemical composition of lipid polyester (i.e. suberin monomers) over the time course of induced *MYB53* overexpression. Tables 1 and 2 report fold-change induction relative to the corresponding mock control at each time-point, whereas Appendix Tables S1 and S2 report the absolute amounts of each lipid polyester monomer for both mock and treated at each time point. First, the monomers were grouped based on common chemistry/biosynthetic origin: ferulate, C20-24 fatty acids, dicarboxylic fatty acids, ω-hydroxy fatty acids, and primary fatty alcohols (Table 3.1). In roots of *MYB53* overexpressing seedlings, the amounts of all these chemical groupings of monomers were significantly increased, between 2.99 and 5.79-fold, compared to the mock-treated seedlings on the 4th day after steroid treatment (Table 3.1). This indicates that *MYB53* overexpression caused an increase of all suberin monomer chemical types, rather than just select chemical types. Ferulate induction compared to mock control decreased on day 7 (4.39 fold induction) compared with day 4 (5.79 fold induction), while C20-C24 fatty acids, dicarboxylic fatty acids and ω-hydroxy fatty acids remained at about the same level of relative induction (4.27 to 4.58 fold induction) and then decreased by day 10 (3.12 to 3.47 fold induction). Primary fatty alcohol induction relative to mock control remained about the same over days 4, 7 and 10 (2.99, 3.61, and 3.38 fold induction, respectively). Additionally, based on multiple relative comparisons of individual monomers at each time-point (Table 3.2), the relative

fold inductions of the majority of individual monomers were, in general, highest on day 4 and remained at almost the same level of induction at day 7. Only ferulate, C22:0 ω-hydroxy fatty acid and C22:0 dicarboxylic fatty acid had statistically significant increases at day 1, but only marginal inductions in each case (1.37, 1.40 and 1.53 fold, respectively). In general, the fold inductions were lower at day 10 compared with day 7, but all monomers remained significantly induced compared to mock control. However, the relative quantities of C18:0, C20:0, and C22:0 primary fatty alcohols showed a different pattern along the four time-point treatment: these three monomers were all significantly increased to maximum levels on day 4 or day 7 after treatment but did not show any significant decrease at day 10 (Table 3.2).

Table 3. 1. Specific groupings based on chemical type of lipid polyester monomers (suberin / cutin) in roots and leaves of the *MYB53* inducible overexpression line over a time-course.

Data is expressed as mean fold change values ± SE, n=3 replicates. Fold change is calculated as the monomer content from β-estradiol treated seedlings relative to the corresponding mock control seedlings (DMSO alone). The red-colored data represents statistical significance, compared pair-wise between mock control and treated group ($p<0.05$, LSD comparison). The small letters show the significant differences among the treatments at all 4 time-points ($p<0.05$, Tukey multiple comparison).

Specific Groupings of Lipid Polyester Components (Suberin/cutin)	Roots				Leaves			
	1 day treatment (11 days growth)	4 days treatment (14 days growth)	7 days treatment (17 days growth)	10 days treatment (20 days growth)	1 day treatment (11 days growth)	4 days treatment (14 days growth)	7 days treatment (17 days growth)	10 days treatment (20 days growth)
Ferulate	1.37 ± 0.07 a	5.79 ± 0.26 c	4.39 ± 0.06 b	3.88 ± 0.31 b	1.05 ± 0.13 a	2.34 ± 0.11 a	12.05 ± 1.65 b	2.08 ± 0.24 a
C20-C24 Fatty Acid Methyl Esters	1.17 ± 0.05 a	4.50 ± 0.07 c	4.27 ± 0.19 c	3.24 ± 0.09 b	1.13 ± 0.15 a	2.68 ± 0.22 b	3.09 ± 0.09 b	3.81 ± 0.34 c
Dicarboxylic Fatty Acid Methyl Esters	1.15 ± 0.04 a	4.57 ± 0.05 c	4.25 ± 0.26 c	3.12 ± 0.11 b	1.00 ± 0.14 a	1.67 ± 0.19 b	1.49 ± 0.09 b	1.84 ± 0.07 b
ω-Hydroxyl Fatty Acid Methyl Esters	1.22 ± 0.02 a	5.00 ± 0.08 c	4.58 ± 0.33 c	3.47 ± 0.13 b	1.07 ± 0.15 a	2.80 ± 0.28 b	2.94 ± 0.22 bc	3.81 ± 0.47 c
Primary Fatty Alcohols	1.01 ± 0.08 a	2.99 ± 0.16 b	3.61 ± 0.10 b	3.38 ± 0.04 b	1.19 ± 0.04 a	1.20 ± 0.17 a	1.40 ± 0.12 a	1.17 ± 0.12 a

Table 3. 2. Lipid polyester (suberin / cutin) monomer composition in roots and leaves of the *MYB53* inducible overexpression line over a time-course.

Data is expressed as mean fold change values ± SE, n=3 replicates. Fold change is calculated as the monomer content from β-estradiol treated seedlings relative to the corresponding mock control seedlings (DMSO alone). The red-colored data represent statistical significance, compared pair-wise between mock control and treated group ($p<0.05$, LSD comparison). The small letters show the significant differences among the treatments at all 4 time-points ($p<0.05$, Tukey multiple comparison).

Lipid Polyester Components (Suberin/Cutin)	Root				Leaf			
	1 day treatment (11 days growth)	4 days treatment (14 days growth)	7 days treatment (17 days growth)	10 days treatment (20 days growth)	1 day treatment (11 days growth)	4 days treatment (14 days growth)	7 days treatment (17 days growth)	10 days treatment (20 days growth)
Hydroxycinnamic Acid Methyl Esters								
Ferulate	1.37 ± 0.03 a	5.79 ± 0.26 c	4.39 ± 0.06 b	3.89 ± 0.31 b	1.05 ± 0.13 a	2.34 ± 0.11 a	12.05 ± 1.65 b	2.08 ± 0.24 a
Fatty Acid Methyl Esters								
C20:0	1.10 ± 0.03 a	4.81 ± 0.03 c	3.97 ± 0.25 bc	3.17 ± 0.11 b	1.05 ± 0.05 a	2.60 ± 0.23 b	2.47 ± 0.10 b	3.29 ± 0.37 b
C22:0	1.16 ± 0.06 a	4.08 ± 0.06 c	3.81 ± 0.15 c	2.87 ± 0.06 b	1.17 ± 0.16 a	3.00 ± 0.26 b	3.82 ± 0.18 bc	4.40 ± 0.50 c
C24:0	1.27 ± 0.06 a	5.55 ± 0.17 c	6.01 ± 0.27 c	4.45 ± 0.18 b	1.09 ± 0.21 a	2.09 ± 0.19 b	2.35 ± 0.09 bc	3.03 ± 0.19 c
Dicarboxylic Fatty Acid Dimethyl Esters								
C16:0	1.12 ± 0.03 a	4.46 ± 0.05 c	3.86 ± 0.22 b	2.89 ± 0.10 b	1.00 ± 0.08 a	1.96 ± 0.18 b	1.66 ± 0.06 b	2.02 ± 0.11 b
C18:2	1.18 ± 0.06 a	3.59 ± 0.25 c	3.57 ± 0.55 c	2.63 ± 0.03 b	0.98 ± 0.17 a	1.31 ± 0.19 a	1.00 ± 0.08 a	1.13 ± 0.05 a
C18:1	1.16 ± 0.04 a	4.61 ± 0.06 c	4.42 ± 0.24 c	3.04 ± 0.07 b	1.07 ± 0.11 a	2.59 ± 0.23 b	2.65 ± 0.17 bc	3.73 ± 0.69 c
C18:0	1.08 ± 0.03 a	4.46 ± 0.05 c	3.78 ± 0.24 b	3.29 ± 0.12 b	1.06 ± 0.22 a	1.58 ± 0.02 ab	1.55 ± 0.09 ab	2.58 ± 1.31 b
C20:0	1.09 ± 0.05 a	4.33 ± 0.03 c	3.57 ± 0.23 b	3.24 ± 0.31 b	0.96 ± 0.31 a	2.06 ± 0.29 bc	1.88 ± 0.13 b	2.98 ± 0.44 c
C22:0	1.53 ± 0.04 a	7.73 ± 0.35 c	6.47 ± 0.37 b	6.52 ± 1.11 b	0.97 ± 0.07 a	3.59 ± 0.42 b	5.81 ± 2.01 c	4.25 ± 1.79 b
ω-Hydroxyl Fatty Acid Methyl Esters								
C16:0	1.23 ± 0.03 a	5.66 ± 0.08 c	5.07 ± 0.38 c	3.80 ± 0.14 b	1.08 ± 0.10 a	2.98 ± 0.28 bc	2.62 ± 0.18 b	3.50 ± 0.45 c
C18:2	1.26 ± 0.06 a	4.46 ± 0.01 c	3.89 ± 0.32 c	2.88 ± 0.05 b	1.02 ± 0.18 a	1.71 ± 0.19 b	1.66 ± 0.16 b	1.82 ± 0.10 b
C18:1	1.19 ± 0.04 a	5.01 ± 0.08 c	4.67 ± 0.35 c	3.35 ± 0.09 b	1.15 ± 0.11 a	3.18 ± 0.33 b	3.19 ± 0.22 b	4.29 ± 0.71 c
C18:0	1.13 ± 0.04 a	4.09 ± 0.06 c	2.77 ± 0.19 b	3.20 ± 0.18 b	1.10 ± 0.15 a	2.13 ± 0.11 b	2.13 ± 0.33 b	3.00 ± 0.09 c
C20:0	1.12 ± 0.03 a	4.29 ± 0.01 c	3.39 ± 0.27 b	3.36 ± 0.25 b	1.11 ± 0.20 a	2.96 ± 0.43 b	2.12 ± 0.17 b	4.15 ± 0.24 c
C22:0	1.40 ± 0.06 a	5.07 ± 0.12 c	4.89 ± 0.29 c	3.55 ± 0.10 b	1.11 ± 0.26 a	3.32 ± 0.34 b	4.43 ± 0.24 bc	4.91 ± 0.58 c
C24:0	0.97 ± 0.47 a	5.27 ± 0.28 bc	5.57 ± 0.36 c	4.43 ± 0.04 b	0.71 ± 0.21 a	1.86 ± 0.40 b	3.18 ± 0.43 bc	3.65 ± 0.94 c
Primary Fatty Alcohols								
C18:0	1.01 ± 0.03 a	3.79 ± 0.08 b	4.09 ± 0.33 b	3.76 ± 0.04 b	1.04 ± 0.11 a	1.94 ± 0.15 b	2.20 ± 0.13 bc	3.03 ± 0.21 c
C20:0	0.91 ± 0.17 a	1.74 ± 0.59 a	3.15 ± 0.39 b	2.99 ± 0.02 b	1.23 ± 0.03 a	1.09 ± 0.18 a	1.05 ± 0.07 a	0.96 ± 0.11 a
C22:0	1.11 ± 0.09 a	3.09 ± 0.09 b	3.32 ± 0.14 b	3.17 ± 0.10 b	1.10 ± 0.29 a	1.81 ± 0.27 ab	2.07 ± 0.17 b	3.46 ± 0.26 c

Leaves are coated in cuticle, which consists of cutin polyester and embedded waxes (Yeats and Rose, 2013). Cutin and suberin are chemically similar glycerol-lipid polymers with some monomers in common. However, they have distinct features. For example, Arabidopsis cutin is made up of about 50% 18:2 monomers, mostly dicarboxylic acids, whereas Arabidopsis suberin consists only of about 2% 18:2 monomers and instead contains high amounts of 18:1 monomers, of which more are ω-hydroxy acids than dicarboxylic acids (Franke *et al.*, 2005). Suberin also contains both long-chain (C16 and C18) and very-long-chain (⩾C20) aliphatics, whereas cutin consists of almost exclusively C16 and C18 chain-length aliphatics. Also, suberin contains ferulate and primary fatty alcohols, which, at least in Arabidopsis, are very low in cutin. Thus, these diagnostic monomers can be used to determine whether suberin is produced in leaves after *MYB53* overexpression, even though the lipid polyester monomers detected here will be of mixed cutin and suberin origin (Kosma *et al.*, 2014). In leaves of plants overexpressing *MYB53*, the amounts of most chemical groupings of monomers (excluding the group of primary fatty alcohols) increased significantly, between 1.67 and 2.80 fold on the 4th day after treatment in comparison to mock (Table 3.1). In leaves, only the relative fold change of ferulate further increased to very high degree on the 7th day after treatment (2.34 fold induction at day 4 and 12.05 fold induction at day 7) and then the fold induction of ferulate decreased to 2.08 on day 10. The relative fold

inductions of C20-C24 fatty acids, dicarboxylic fatty acids and ω-hydroxy fatty acids were all maintained at steady levels over days 4, 7 and 10, with C20-C24 fatty acids and ω-hydroxy fatty acids having their maximum fold inductions at day 10 (both 3.81 fold inductions at day 10). The relative fold inductions of primary fatty alcohols did not change much along the entire time-course, and were very modestly induced (1.40 fold at day 7 being the maximum). The majority of individual monomers significantly increased starting at day 4 after *MYB53* overexpression and then remained increased through day 10. The exceptions were C18:2 dicarboxylic acid, which is a cutin diagnostic monomer, and C20:0 primary alcohol, of which neither displayed a significant increase in leaf lipid polyester over the entire time-course.

3.2 Upregulation of suberin biosynthetic genes in roots and leaves by steroid-induced overexpression of Arabidopsis *MYB53*

Since *MYB53* overexpression caused significant accumulation of suberin in both Arabidopsis roots and leaves, including all monomer types, we expected that *MYB53* overexpression caused the upregulation of all suberin biosynthetic genes. Since *MYB53* was already obviously overexpressed 16 hrs after β-estradiol treatment (Figure 3.2A and 3.2B), we therefore investigated the transcript levels of suberin biosynthetic genes at this same time point in both roots and leaves. This was done by quantitative RT-PCR (Figures

3.4 and 3.5). All suberin biosynthetic genes tested (*CYP86A1*, *CYP86B1*, *GPAT5*, *ASFT*, *FAR1*, *FAR4*, and *FAR5*) were significantly up-regulated in both roots and leaves 16 hours after adding steroid. In general, suberin biosynthetic genes were more highly induced in roots than in the leaves. *CYP86A1* (20.13 fold), *CYP86B1* (12.56 fold) and *GPAT5* (12.36 fold) were the three most up-regulated genes in roots, and the other four genes in roots were up-regulated between 1.21 and 6.69 fold. In leaves, *CYB86B1* (7.91 fold), *ASFT* (6.90 fold) and *GPAT5* (3.78 fold) were the three most up-regulated genes, and the other four genes in leaves were up-regulated between 1.18 and 1.68 fold.

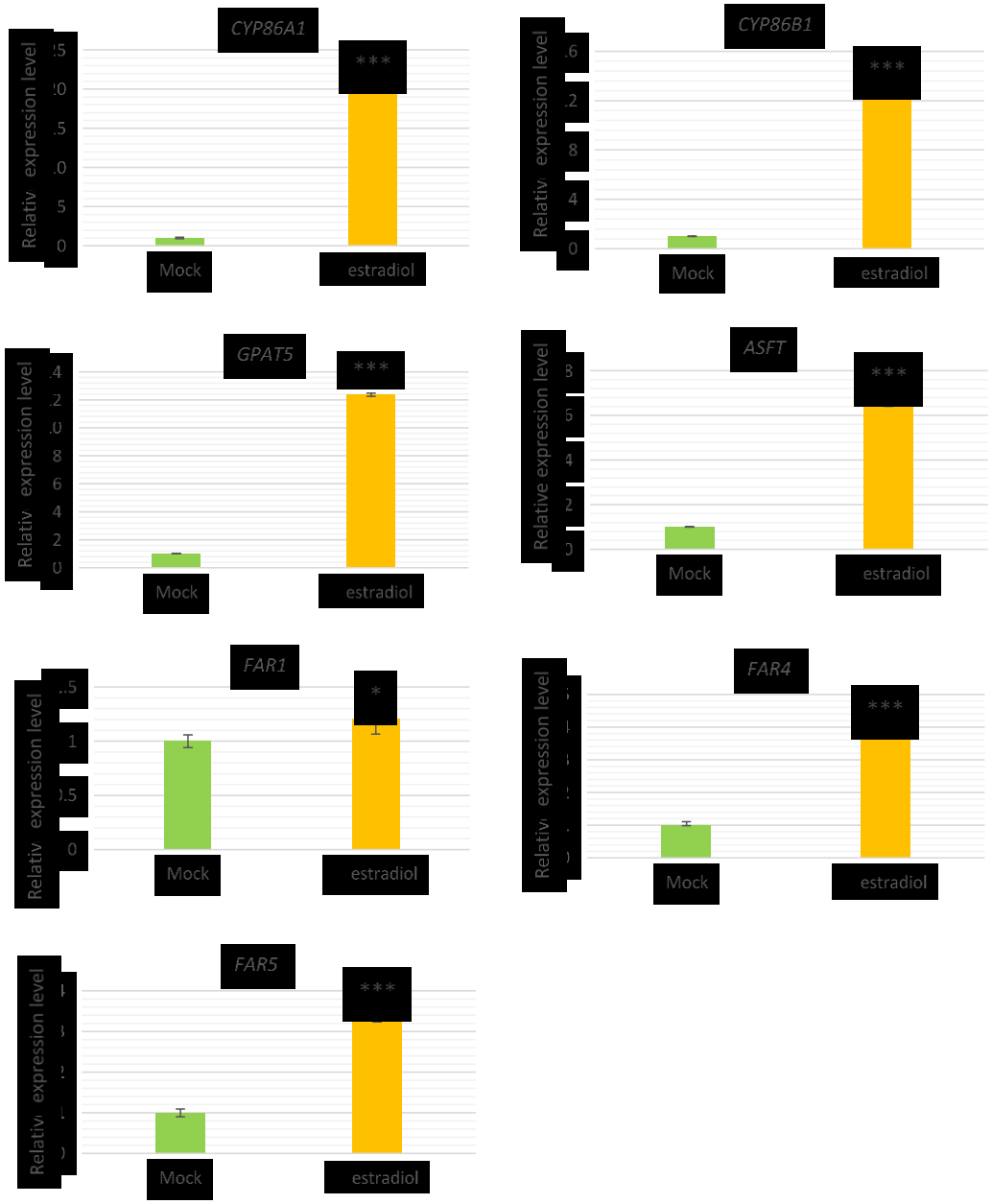


Figure 3.4. Quantitative RT-PCR analysis of suberin biosynthetic gene transcripts in roots of the *MYB53* inducible overexpression line from β-estradiol treated and mock control (DMSO alone) seedlings.

The seedlings were 10-days old when treated with β-estradiol or DMSO solvent only, and then roots were harvested 16 hours later for RNA analysis. Data are presented as mean fold change values ± SE from 3 biological replicates and 2 technical replicates of each biological replicate. Approximately the root tissue of 50 seedlings were pooled to make one biological replicate. The asterisks represent the statistical significance, compared

pair-wise between mock control and treated groups ("*" represents $p<0.05$, "**" represents $p<0.01$, "***" represents $p<0.001$, Student's t-test, one-tailed).

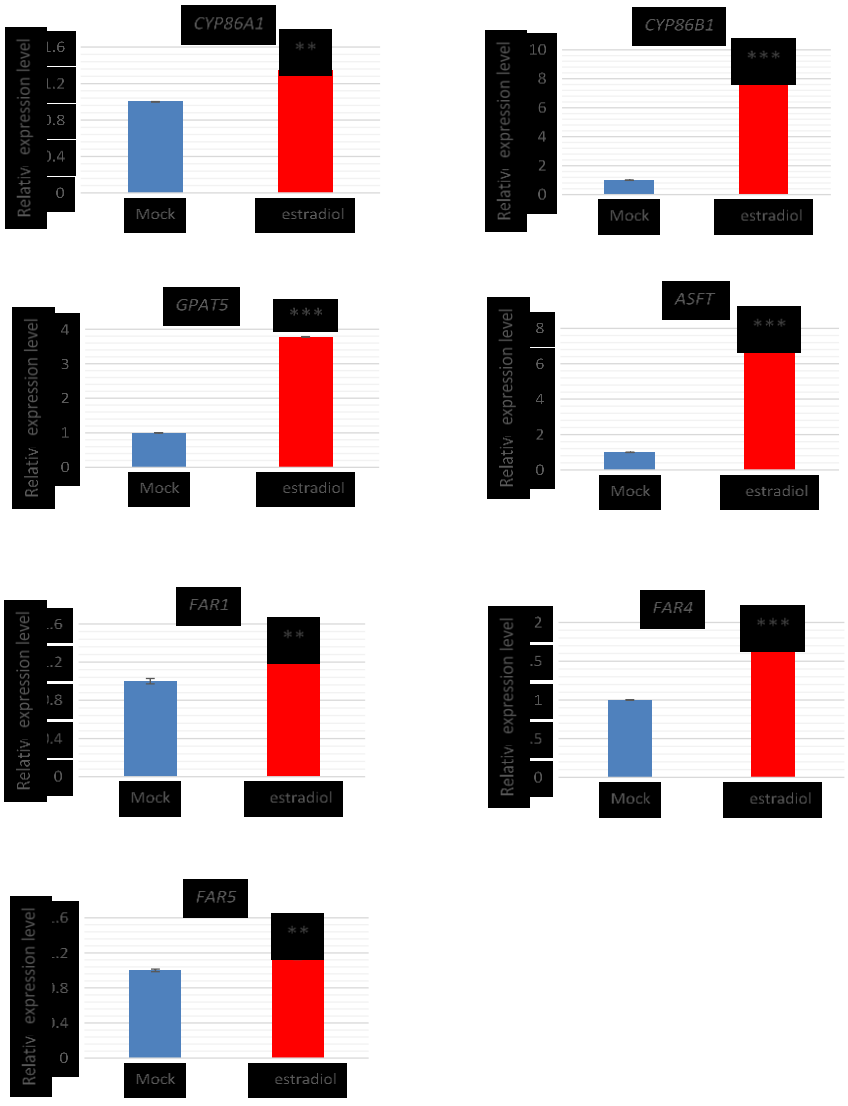


Figure 3.5. Quantitative RT-PCR analysis of suberin biosynthetic gene transcripts in leaves of the *MYB53* inducible overexpression line from β-estradiol treated and mock control (DMSO alone) seedlings.

The seedlings were 10-days old when treated with β-estradiol or DMSO solvent only, and then leaves were harvested 16 hours later for RNA analysis. Data are presented as mean fold change values ± SE from 3 biological replicates and 2 technical replicates of each biological replicate. Approximately the root tissue of 50 seedlings were pooled to make one biological replicate. The asterisks represent the statistical significance, compared pair-wise between mock control and treated groups ("*" represents $p<0.05$, "**" represents $p<0.01$, "***" represents $p<0.001$, Student's t-test, one-tailed).

3.3 Molecular identification of *MYB53/92/93* loss-of-function single, double and triple mutant lines

Two independent *myb53* T-DNA mutants, *myb53-1* and *myb53-2*, were crossed with a set of previously characterized *myb92-1*, *myb93-1*, and *myb92-1 myb93-1* mutants (Murmu *et al.*, Rowland lab, manuscript in preparation) to create here two collections of *MYB53/92/93* single, double and triple loss-of-function mutants. The T-DNA insertion in *myb53-1* was at the beginning of the third exon, while the T-DNA in *myb53-2* was at the end of third exon (Figure 3.6A). The T-DNA in *myb92-1* was at the end of the second exon and the T-DNA in *myb93-1* was at the beginning of the first exon (Figure 3.6A). Multiple pairs of primers were designed to flank each T-DNA insertion. Two pairs of primers, F1/R1 and F2/R2, were designed to flank the T-DNA insertions of *myb53-1* and *myb53-2*, respectively. Similarly, the primer pairs, F3/R3 and F4/R4, were designed to flank the corresponding T-DNA insertion sites of *myb92-1* and *myb93-1*, respectively. Semi-quantitative RT-PCR using RNA isolated from roots was used to examine the abundance of *MYB53/MYB92/MYB93* transcript in every single, double, and triple mutant compared with wild-type (Figure 3.6B). It was observed that *MYB53* transcript was PCR amplified in *myb53-2, myb53-2 myb92-1, myb53-2 myb93-1* and *myb53-2 myb92-1 myb93-1* lines when using *MYB53* primers F1 and R1, which anneal upstream of the T-DNA insertion site in *myb53-2*. However, when using F2 and R2, which anneal

downstream and flanking the T-DNA insertion site of *myb53-2,* there was no amplification of *MYB53* in any *myb53-1/myb53-2* single, double or triple mutants (Figure 3.6B). There was no amplification of *MYB92* or *MYB93* in *myb92-1* or *myb93-1* single, double or triple mutants, when using primers F3/R3 or F4/R4, respectively. In total, these results indicate that all *myb* mutants are likely knock-out loss-of-function lines, but partial transcripts may be produced, at least in *myb53-2*.

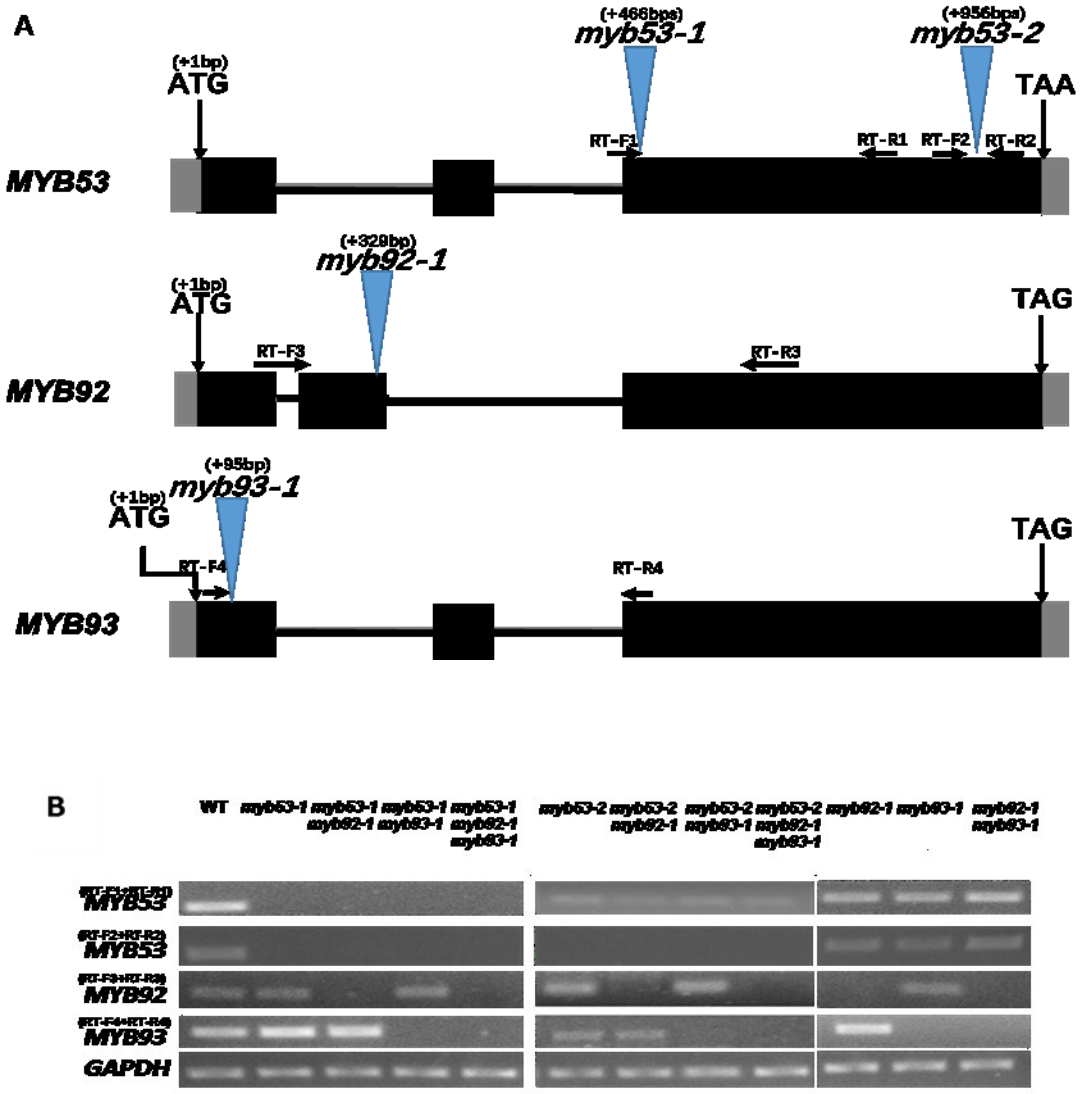


Figure 3.6. *MYB53* (At5g65230), *MYB92* (At5g10280), and *MYB93* (At1g34670) gene structures and transcript levels in *myb53/myb92/myb93* loss-of-function lines.
(A) Schematic representations of the *MYB53, MYB92,* and *MYB93* gene structures, including exons (black boxes), introns (black lines), and the 5'- and 3'- untranslated regions (gray boxes). The position where the T-DNA is inserted is indicated with blue triangle for each mutant allele, *myb53-1, myb53-2, myb92-1* and *myb93-1*, with the upstream T-DNA insertion site indicated in base pairs relative to the A (+1) of the start codon. The primer annealing sites used for RT-PCR are also indicated. (B) RT-PCR analysis of *MYB53, MYB92,* and *MYB93* transcript levels of the entire collection of *loss-of-function* single, double and triple mutants compared with wild-type using primer pairs individually spanning each T-DNA insertion site as indicated by the arrows in panel A. *GAPDH* (At1g13440) was used as the constitutive reference gene.

3.4 Decreased suberin content in *MYB53/92/93* loss-of-function mutants

The root suberin content in wild-type and all mutants was determined by gas chromatography analysis of suberin monomers after depolymerization of the polymer via base-catalyzed transmethylation. The seedlings of all mutants were grown in tissue culture plates for 2 weeks of growth. Because of the technical difficulties of growing and harvesting all 11 mutants at the same time, these mutants were grown in four batches and in each batch wild-type control was also grown for comparison. The quantities of total suberin polymer and suberin monomers for each mutant were reported as the relative abundance to wild-type grown at the same time (absolute values are reported in Appendix Tables). All 11 single, double and triple mutants had significantly decreased total suberin compared with the wild-type (Figure 3.7). Compared with wild-type, *myb53-1*, *myb53-2*, *myb92-1* and *myb93-1* single mutants displayed decreased suberin amounts of 19-29%. Double mutants *myb53-1 myb92-1, myb53-1 myb93-1, myb53-2 myb92-1, myb53-2 myb93-1* and *myb92-1 myb93-1* had reduced suberin amounts of 39%-57%, with *myb92-1 myb93-1* being the most decreased amongst the double mutants. Compared with wild-type, the total suberin content of the two triple *myb53 myb92 myb93* mutants were severely decreased, each by about 72%.

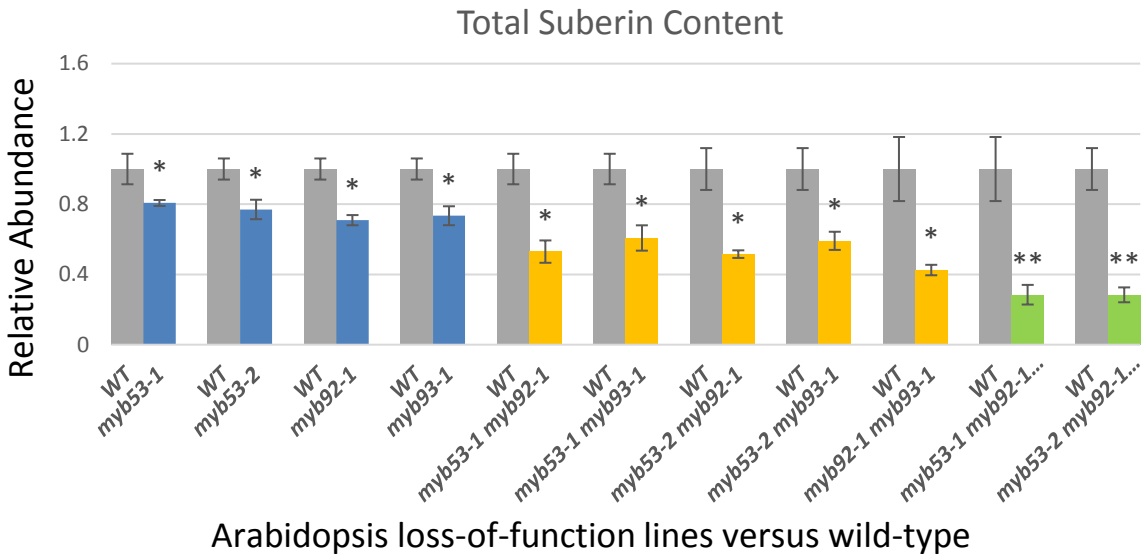


Figure 3.7. Total suberin content in roots from 2-week-old seedlings of the MYB53/MYB92/MYB93 *loss–of–function* single, double and triple mutant lines compared with the corresponding wild-type (WT).

For wild-type and each mutant, root tissue from approximately 180 seedlings was pooled to make one biological replicate and three biological replicates were made. This graph represents multiple batches of experiments completed at separate times: data shown in the graph are represented as the average values relative to respective wild-type value set at 1.0 (error bars = SE). This was because of the operational difficulties of growing, harvesting and processing seedlings of all 11 mutants and the wild-type in one batch within one day and there is variation in the absolute suberin amounts when grown on separate dates. The asterisks indicate the significant difference of each line pairwise compared with its respective wild-type control ("*" represents $p<0.05$, "**" represents $p<0.01$, LSD comparison).

Further detailed analysis of suberin composition in the mutants was also conducted. First, a direct comparison between the *myb53-1* and *myb53-2* alleles was done, specifically comparing *myb53-1* with *myb53-2, myb53-1 myb92-1* with *myb53-2 myb92-1, myb53-1 myb93-1* with *myb53-2 myb93-1,* and *myb53-1 myb92-1 myb93-1* with *myb53-2 myb92-1 myb93-1* in terms of both suberin monomer composition and the total suberin. There were no significant differences between the two *myb53* allele collections (Appendix B, Table S7). They are likely both knock-out loss-of-function alleles.

In terms of individual monomers (Table 3.3), the single mutants *myb53-1, myb53-2, myb92-1* and *myb93-1* only showed significant reductions in 3-7 suberin monomer types compared with wild-type. Only C18:1 ω-hydroxy fatty acids were significantly reduced in all four single mutants. The double mutants *myb53-1 myb92-1, myb53-1 myb93-1, myb53-2 myb92-1* and *myb53-2 myb93-1* had significant reductions in 11-17 suberin monomer types, with *myb53-1 myb92-1* and *myb53-2 myb92-1* affected in more types of monomers than *myb53-1 myb93-1* and *myb53-2 myb93-1*. The double mutant *myb92-1 myb93-1* and the triple mutants *myb53-1 myb92-1 myb93-1* and *myb53-2 myb92-1 myb93-1* had significant reductions in all suberin monomers.

Table 3. 3. Main suberin monomers in roots from 2-weeks-old seedlings of the *MYB53/MYB92/MYB93 loss–of–function* single, double and triple mutant lines.

For wild-type and each mutant, the root tissue from approximately 180 seedlings was pooled to make one biological replicate and three biological replicates were made. The data are expressed by mean fold change values ± SE relative to the wild-type control grown at the same time. Suberin analyses of these 11 mutant lines were completed separately in 4 batches, each time with wild-type present for comparison. Fold change is calculated as the suberin monomer content from each mutant allele relative to the wild-type, set at 1.00, in each batch of experiments. The red-coloured data have a significant reduction compared with the respective wild-type control ($p<0.05$, LSD comparison).

Relative Abundance	*myb53-1*	*myb53-2*	*myb92-1*	*myb93-1*	*myb53-1 myb92-1*	*myb53-1 myb93-1*	*myb53-2 myb92-1*	*myb53-2 myb93-1*	*myb92-1 myb93-1*	*myb53-1 myb92-1 myb93-1*	*myb53-2 myb92-1 myb93-1*
Hydroxycinnamic Acid Methyl Esters											
Ferulate	0.88 ± 0.08	0.87 ± 0.08	0.87 ± 0.07	0.81 ± 0.02	0.74 ± 0.14	0.72 ± 0.13	0.71 ± 0.17	0.65 ± 0.04	0.63 ± 0.03	0.49 ± 0.09	0.63 ± 0.05
Fatty Acid Methyl Esters											
C20:0	0.89 ± 0.06	0.80 ± 0.07	0.77 ± 0.08	0.65 ± 0.05	0.68 ± 0.16	0.78 ± 0.20	0.71 ± 0.01	0.77 ± 0.13	0.48 ± 0.03	0.31 ± 0.07	0.36 ± 0.08
C22:0	0.90 ± 0.01	0.72 ± 0.12	0.74 ± 0.02	0.81 ± 0.12	0.46 ± 0.08	0.53 ± 0.04	0.49 ± 0.06	0.53 ± 0.05	0.36 ± 0.03	0.22 ± 0.04	0.30 ± 0.03
C24:0	0.84 ± 0.03	0.84 ± 0.07	0.79 ± 0.15	0.67 ± 0.11	0.70 ± 0.15	0.80 ± 0.16	0.70 ± 0.10	0.79 ± 0.10	0.33 ± 0.03	0.25 ± 0.04	0.48 ± 0.16
Dicarboxylic Fatty Acid Dimethyl Esters											
C16:0	0.90 ± 0.04	0.81 ± 0.07	0.70 ± 0.07	0.71 ± 0.09	0.53 ± 0.05	0.65 ± 0.05	0.57 ± 0.03	0.64 ± 0.12	0.44 ± 0.03	0.29 ± 0.06	0.33 ± 0.02
C18:2	0.82 ± 0.01	0.80 ± 0.14	0.58 ± 0.05	0.77 ± 0.09	0.47 ± 0.08	0.57 ± 0.07	0.50 ± 0.03	0.53 ± 0.17	0.41 ± 0.02	0.27 ± 0.05	0.26 ± 0.10
C18:1	0.62 ± 0.03	0.74 ± 0.06	0.62 ± 0.01	0.74 ± 0.03	0.43 ± 0.03	0.49 ± 0.05	0.48 ± 0.04	0.42 ± 0.02	0.35 ± 0.03	0.18 ± 0.06	0.14 ± 0.01
C18:0	0.84 ± 0.07	0.75 ± 0.09	0.76 ± 0.11	0.59 ± 0.03	0.56 ± 0.08	0.69 ± 0.16	0.51 ± 0.05	0.64 ± 0.11	0.44 ± 0.04	0.27 ± 0.05	0.31 ± 0.08
C20:0	0.78 ± 0.07	0.71 ± 0.07	0.87 ± 0.14	0.48 ± 0.07	0.56 ± 0.09	0.66 ± 0.23	0.57 ± 0.07	0.66 ± 0.07	0.50 ± 0.06	0.27 ± 0.05	0.33 ± 0.08
C22:0	0.87 ± 0.09	0.80 ± 0.08	0.74 ± 0.15	0.98 ± 0.16	0.64 ± 0.06	0.76 ± 0.12	0.65 ± 0.12	0.75 ± 0.09	0.53 ± 0.05	0.35 ± 0.07	0.59 ± 0.08
ω-Hydroxyl Fatty Acid Methyl Esters											
C16:0	0.84 ± 0.04	0.71 ± 0.06	0.76 ± 0.05	0.70 ± 0.07	0.62 ± 0.11	0.73 ± 0.19	0.65 ± 0.03	0.72 ± 0.11	0.53 ± 0.03	0.36 ± 0.08	0.33 ± 0.08
C18:2	0.79 ± 0.02	0.69 ± 0.02	0.70 ± 0.05	0.72 ± 0.07	0.47 ± 0.08	0.58 ± 0.09	0.50 ± 0.01	0.60 ± 0.01	0.37 ± 0.02	0.22 ± 0.04	0.22 ± 0.04
C18:1	0.79 ± 0.03	0.71 ± 0.05	0.64 ± 0.02	0.68 ± 0.02	0.46 ± 0.02	0.50 ± 0.02	0.43 ± 0.01	0.54 ± 0.02	0.34 ± 0.03	0.18 ± 0.04	0.19 ± 0.01
C18:0	0.80 ± 0.06	0.73 ± 0.08	0.79 ± 0.08	0.58 ± 0.02	0.67 ± 0.07	0.78 ± 0.25	0.66 ± 0.02	0.74 ± 0.13	0.53 ± 0.06	0.34 ± 0.08	0.34 ± 0.08
C20:0	0.92 ± 0.04	0.96 ± 0.13	0.71 ± 0.13	0.74 ± 0.13	0.72 ± 0.17	0.85 ± 0.32	0.71 ± 0.10	0.89 ± 0.13	0.40 ± 0.04	0.30 ± 0.07	0.38 ± 0.06
C22:0	0.81 ± 0.04	0.81 ± 0.07	0.85 ± 0.13	0.98 ± 0.10	0.60 ± 0.09	0.71 ± 0.17	0.58 ± 0.07	0.71 ± 0.07	0.52 ± 0.06	0.31 ± 0.06	0.40 ± 0.07
C24:0	0.73 ± 0.01	0.73 ± 0.09	0.64 ± 0.08	0.96 ± 0.11	0.44 ± 0.08	0.47 ± 0.11	0.40 ± 0.05	0.43 ± 0.03	0.37 ± 0.05	0.20 ± 0.04	0.33 ± 0.06
Primary Fatty Alcohols											
C18:0	0.98 ± 0.09	0.98 ± 0.06	0.78 ± 0.05	0.60 ± 0.06	0.71 ± 0.13	0.85 ± 0.26	0.69 ± 0.05	0.83 ± 0.12	0.71 ± 0.04	0.57 ± 0.14	0.66 ± 0.24
C20:0	0.97 ± 0.04	0.98 ± 0.16	0.76 ± 0.06	0.69 ± 0.08	0.55 ± 0.18	0.68 ± 0.07	0.53 ± 0.01	0.65 ± 0.12	0.44 ± 0.03	0.30 ± 0.06	0.35 ± 0.23
C22:0	0.98 ± 0.07	0.97 ± 0.08	0.70 ± 0.06	0.73 ± 0.09	0.37 ± 0.08	0.44 ± 0.18	0.36 ± 0.02	0.40 ± 0.03	0.35 ± 0.04	0.25 ± 0.04	0.33 ± 0.06

Additionally, we investigated the relationship between MYB53/92/93 mutants and effects on suberin composition grouped into chemical types (Table 3.4). Again, the groupings were based on chemistry / biosynthetic origin as follows: ferulate, C20-24 fatty acids, dicarboxylic fatty acids, ω-hydroxy fatty acids, and primary fatty alcohols. Generally, all chemical classes were decreased the most severely in the two triple *myb53 myb92 myb93* mutants (37%-76% decreases), followed by the double mutants (26%-51% decreases), and then the single mutants (by 2%-34% decreases). The decreased levels of suberin monomer groups in *myb53-1 myb92-1* and *myb53-2 myb92-1* were approximately the sum of the corresponding reductions in the single mutants *myb53-1* or *myb53-2* and *myb92-1* (Table 3.4, Figure 3.7). Also, the total suberin reduction in double mutants *myb53-1 myb93-1* and *myb53-2 myb93-1* was, respectively, 39% and 41%, and in the single mutants *myb53-1, myb53-2* and *myb93-1* was 19%, 23% and 27%, or about additive. Similarly, the reduced levels of ferulate and ω-hydroxy fatty acids were roughly the sum of the reductions in the corresponding single mutants *myb53-1, myb53-2* and *myb93-1*. Third, the total suberin reduction in *myb92-1 myb93-1* (57% decrease) was more severe than either of *myb92-1* and *myb93-1.* Again, it is found that the decreased levels of monomers, including ferulate, dicarboxylic fatty acids, ω-hydroxy fatty acids, and primary fatty alcohols, in *myb92-1 myb93-1* were about the same as adding the individual reductions observed in *myb92-1* and *myb93-1*. Finally, the total suberin in the

triple mutants *myb53-1 myb92-1 myb93-1* and *myb53-2 myb92-1 myb93-1* was each decreased by about 72%, which is a much greater reduction than in any single or double mutant *myb53-1* (~19%) / *myb53-2* (~23%) / *myb92-1* (~29%) / *myb93-1* (~27%) or double mutant *myb53-1 myb92-1* (~47%) / *myb53-2 myb92-1* (~48%) / *myb53-1 myb93-1* (~39%) / *myb53-2 myb93-1* (~41%) / *myb92-1 myb93-1* (~57%). In particular, the reduced levels of dicarboxylic fatty acids, ω-hydroxy fatty acids in the triple mutants were approximately the reduction in any double mutant plus the third *myb* single mutant, or the sum of reduction in all three *myb* single mutants.

Table 3. 4. Specific groupings based on chemical type of main suberin monomers in roots from 2-weeks-old seedlings of the *MYB53/MYB92/MYB93 loss–of–function* single, double and triple mutant lines.

For wild-type and each mutant, the root tissue from approximately 180 seedlings was pooled to make one biological replicate and three biological replicates were made. The data are expressed by mean fold change values ± SE relative to the wild-type control grown at the same time. Suberin analyses of these 11 mutant lines were completed separately in 4 batches, each time with wild-type present for comparison. Fold change is calculated as the suberin monomer content from each mutant relative to the wild-type, set at 1.00, in each batch of experiments. The red-coloured data have a significant reduction compared with the respective wild-type control ($p<0.05$, LSD comparison).

Specific groupings of main suberin monomers	*myb53-1*	*myb53-2*	*myb92-1*	*myb93-1*	*myb53-1 myb92-1*	*myb53-1 myb93-1*	*myb53-2 myb92-1*	*myb53-2 myb93-1*	*myb92-1 myb93-1*	*myb53-1 myb92-1 myb93-1*	*myb53-2 myb92-1 myb93-1*
Ferulate	0.88 ± 0.08	0.87 ± 0.08	0.87 ± 0.07	0.81 ± 0.02	0.74 ± 0.14	0.72 ± 0.13	0.71 ± 0.17	0.65 ± 0.04	0.63 ± 0.03	0.49 ± 0.09	0.63 ± 0.05
C20-C24 Fatty Acid Methyl Esters	0.89 ± 0.02	0.75 ± 0.09	0.75 ± 0.02	0.76 ± 0.10	0.54 ± 0.10	0.61 ± 0.07	0.56 ± 0.06	0.61 ± 0.07	0.37 ± 0.03	0.24 ± 0.04	0.34 ± 0.06
Dicarboxylic Fatty Acid Methyl Esters	0.74 ± 0.02	0.76 ± 0.05	0.66 ± 0.04	0.72 ± 0.05	0.49 ± 0.05	0.57 ± 0.07	0.50 ± 0.04	0.56 ± 0.04	0.41 ± 0.03	0.25 ± 0.05	0.24 ± 0.03
ω-Hydroxyl Fatty Acid Methyl Esters	0.80 ± 0.02	0.73 ± 0.05	0.70 ± 0.03	0.75 ± 0.04	0.49 ± 0.05	0.58 ± 0.08	0.49 ± 0.02	0.58 ± 0.04	0.40 ± 0.04	0.24 ± 0.05	0.26 ± 0.03
Primary Fatty Alcohols	0.98 ± 0.06	0.98 ± 0.03	0.76 ± 0.04	0.66 ± 0.06	0.57 ± 0.13	0.68 ± 0.18	0.56 ± 0.03	0.68 ± 0.09	0.53 ± 0.04	0.40 ± 0.09	0.48 ± 0.18

3.5 Altered expression levels of suberin biosynthetic genes in *MYB53/92/*93 loss-of-function mutants

The transcript levels of seven suberin biosynthetic genes were then investigated in all 11 *myb* loss-of-function mutants in comparison to wild-type (Figure 3.8). The relative expression levels of all these suberin-related genes followed a similar pattern: (1) the *myb* loss-of-function mutants all displayed a lower expression level of target genes than the wild-type; (2) the expression levels in single, double and triple mutants were progressively decreased, with the triple mutants showing the least amount of transcript. These shared features indicate that *MYB53, MYB92* and *MYB93* are all required for the expression of these suberin biosynthetic genes. Additionally, among these suberin-related genes, the transcript levels of *CYP86A1, CYP86B1* and *GPAT5* were the most affected in *the myb* mutants compared with wild-type, with a fold reduction decreases of approximately 5%-70% in the single mutants, 57%-90% in the double mutants, and 85%-96% in the triple mutants. By contrast, the transcript levels of *FAR1, FAR4* and *FAR5* did not decrease as much in the *myb* mutants compared with the wild-type. The fold reduction decreases were 1%-57% in the single mutants, and the expression level of *FAR4* in was even slightly increased by 2% *myb53-2*. In the double and triple mutants, the transcript levels of *FAR1, FAR4,* and *FAR5* were reduced by 30%-77% and 63%-

83%, respectively. The expression level of *ASFT* was also reduced compared with wild-type, although not reaching a statistically significant level, although this may be due to highly variant technical repetitions.

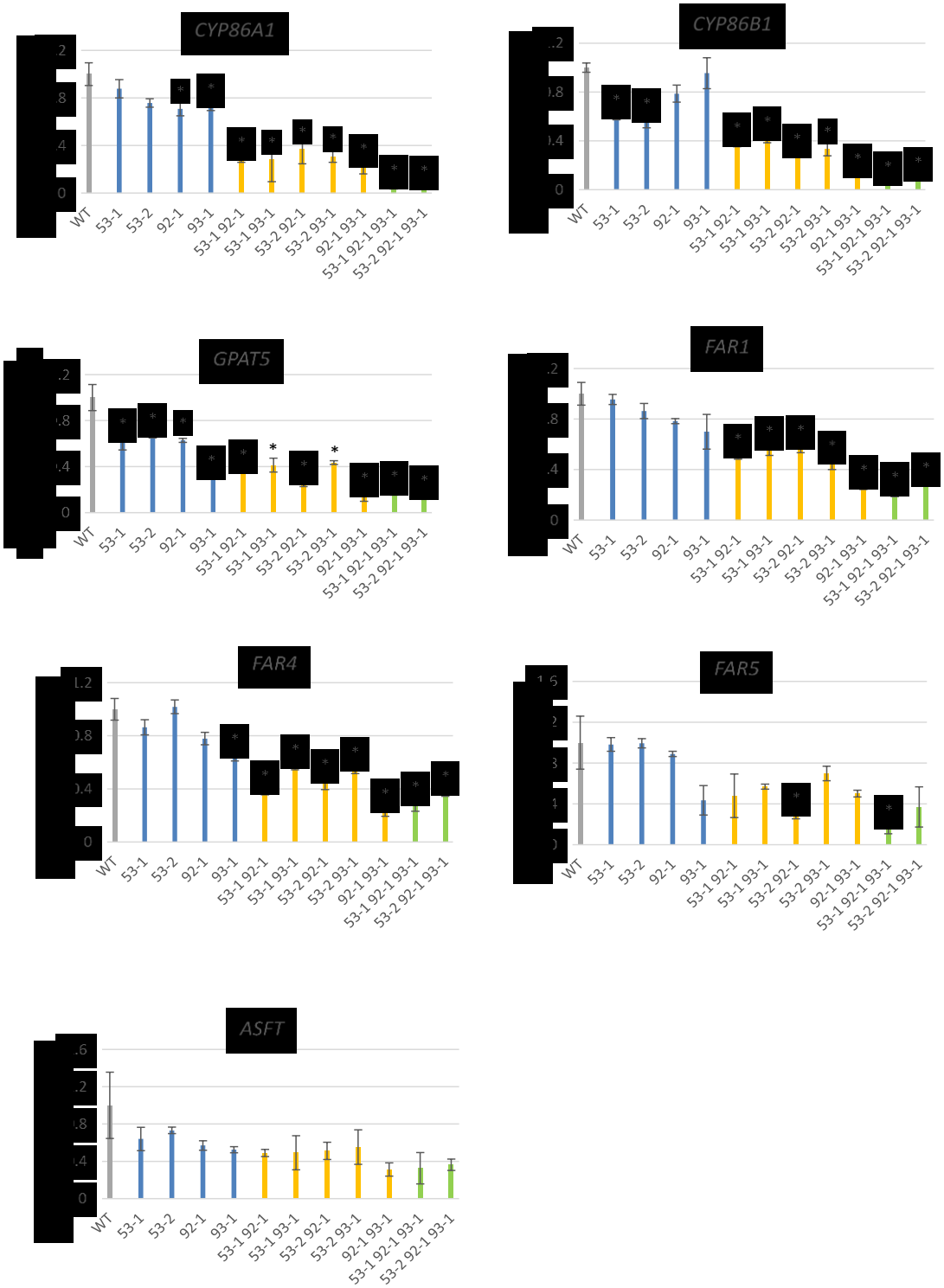


Figure 3.8. Quantitative RT-PCR analysis of suberin biosynthetic genes in roots of 2-week-old seedlings of *MYB53/MYB92/MYB93 loss-of-function* single, double and triple mutant lines compared with wild type (WT).

53-1, 53-2, 92-1, 93-1, 53-1 92-1, 53-2 92-1, 53-1 93-1, 53-2 93-1, 92-1 93-1, 53-1 92-1

93-1 and *53-2 92-1 93-1* represents mutants *myb53-1, myb53-2, myb92-1, myb93-1, myb53-1 myb92-1, myb53-2 myb92-1, myb53-1 myb93-1, myb53-2 myb93-1, myb92-1 myb93-1, myb53-1 myb92-1 myb93-1* and *myb53-2 myb92-1 myb93-1*, respectively. Data is presented as mean fold change values ± SE from 3 biological replicates and 2 technical replicates of each bio-repetition. An "*" indicates significant reduction in the relative expression level of a gene in a mutant compared with wild-type ($P<0.05$, LSD comparison).

Chapter 4: Discussion

4.1 MYB53 positively regulates suberin biosynthesis in Arabidopsis

Transient overexpression of *MYB53* using the steroid inducible TRANSPLANTA Arabidopsis line increased the amount of suberin in both roots and leaves within 4 days of steroid application, while loss of *MYB53* gene activity decreased the amount of root suberin in two independent *myb53 loss-of-function* mutant lines. This means that *MYB53* is positively regulating suberin biosynthesis. Additionally, the *MYB53* mutants in combination with *MYB92* and/or *MYB93* mutants resulted in further reductions of suberin. Among them, the triple *myb53 myb92 myb93 loss-of-function* mutants were most affected, featured with approximately a 72% decrease of suberin in comparison to wild-type. This is the largest reduction of root suberin reported to date in mutants of Arabidopsis (Vishwanath *et al.*, 2015).

MYB53 overexpression and loss-of-function, respectively, resulted in increases and decreases of all suberin monomers in young roots of Arabidopsis. Meanwhile, *MYB53* overexpression also led to ectopic accumulation of all suberin-type monomers in leaves, such that the leaves contained both cutin and suberin lipid-polyesters. These findings, along with the previously reported study showing that Arabidopsis *MYB41* overexpression caused all suberin-like monomers to accumulate in leaves (Kosma *et al*., 2014), are different from a previous study where co-expression of suberin biosynthetic

genes, *GPAT5* and *CYP86A1*, resulted in ectopic deposition of just a few suberin monomer types, such as C20-C22 ω-hydroxy fatty acids and α,ω-dicarboxylic acids (Li *et al*., 2007). Also, the single overexpression of *CYP86A1* caused no chemical change in leaf polyester, and the single overexpression of *GPAT5* only caused ectopic accumulation of very-long-chain fatty acid-containing monoacylglycerols in leaf cuticle waxes (Li *et al*., 2007). In our study, the conclusion that *MYB53* regulates the complete suberin polymer was further supported by our observation that *MYB53* activates transcription of a suite of genes required to synthesize and assemble suberin monomer precursors. Although we have not tested the transcript levels of every suberin-associated gene known to date, such as the genes coding ABC transporters (*ABCG2*, *ABCG6* and *ABCG20*), or predicted genes encoding cell wall-associated polyester synthases, the fact that total suberin polymer was significantly altered by changes in *MYB53* gene activity indicates that MYB53 functions as a master regulator of suberin that switches on all downstream suberin-related genes.

Several other MYB transcription factors have been found to positively regulate suberin biosynthetic genes in Arabidopsis, however these control suberin formation in various other tissues either during normal developmental stages or in response to stresses. Arabidopsis *MYB41* is expressed in the endodermis of young roots, but only under stress conditions involving ABA and high salts (Kosma *et al*., 2014). Whether there is a

mechanistic connection between MYB41 and MYB53 under these conditions is currently unknown (e.g. MYB41 may upregulate MYB53 to turn on downstream suberin genes). In Arabidopsis seed coat, the loss of *MYB9* and *MYB107* gene activities are positively correlated with decreased transcript levels of all tested suberin biosynthetic genes in seeds, with corresponding alterations in seed coat suberin involving all major components (Lashbrooke *et al*., 2016; Gou *et al.*, 2017). A result of this is increased seed coat permeability. The effects of *MYB9* and *MYB107 loss-of-function* mutations were restricted to seeds and no alteration in root suberin were observed. Therefore, there appears to be different MYBs controlling suberin production in the various tissues or conditions where suberin is deposited. *At*MYB107 was further found to directly target suberin-related genes in seed coats of Arabidopsis by binding to their promoters (Gou *et al.*, 2017), but it remains to be tested whether MYB53 directly or indirectly regulates suberin biosynthetic genes in Arabidopsis roots (see Future Directions in Chapter 5).

In our study, only the suberin polymer was analyzed in *MYB53* overexpression and loss-of-function lines, while characterization of soluble lipids, in particular suberin-associated waxes, were not investigated here. Suberin-associated waxes were found to be increased in leaves when *AtMYB41* was stably overexpressed (Kosma *et al*., 2014), and it will be important to investigate if these compounds are also altered when MYB53 gene activity is altered (Chapter 5).

4.2 *MYB53* can regulate suberin deposition in multiple tissue types, but its regulatory control normally occurs in root endodermis

The total amount of suberin accumulated to much higher levels in roots than in leaves after *MYB53* overexpression. It is also known that *MYB53* is normally only expressed in root endodermis using *MYB53 promoter:: GUS* lines (unpublished data, Rowland lab). Thus, it seems that compared with leaves, roots are more responsive to *MYB53* overexpression and/or intrinsically more capable of accumulating suberin. It is currently unclear whether this difference is due to the very high amounts of suberin being deposited in the walls of root endodermis only, which normally makes suberin, or if additional layers of cells are being suberized in roots than that of leaves (Chapter 5).

Suberin and cutin are both cell wall-associated lipid-based polymers that have similar chemical components, but it seems that there is no overlap in the factors regulating these two polymers. In our experiment, suberin-type monomers, but not cutin-type monomers, were increased in leaves after *MYB53* overexpression, indicating that *MYB53* is specifically involved in the suberin biosynthetic pathway. Meanwhile, some transcription factors regulate cutin biosynthesis but not suberin biosynthesis. For example, Arabidopsis *WIN1/SHN1* encodes an AP2/ERF (APETALA 2/ethylene responsive factor)-type transcription factor that positively regulates cutin and associated

wax biosynthesis but not suberin accumulation (Broun *et al.*, 2004; Aharoni *et al.*, 2004; Kannangara *et al*., 2007). *At*WIN1/SHN1 was shown to directly bind the promoter of the *LONG-CHAIN ACYL-CoA SYNTHETASE2* (*LACS2*) gene involved in cutin biosynthesis (Kannangara *et al*., 2007).

We conducted a time course over 10 days to analyze suberin formation after inducing *MYB53* overexpression using the TRANSPLANTA steroid inducible system. It has been shown that the overexpression of target genes is not maintained in such a system due to the instability of β-estradiol (Zuo *et al*., 2000). This instability likely results in loss of *MYB53* overexpression over time, and then less suberin induction as the roots and leaves develop from 4 to 10 days. In roots, the fold inductions of most suberin monomers (except for primary fatty alcohols) were maximized on day 4 after applying steroid. However, in leaves, the fold inductions of most suberin monomers were maximized on day 10. The reason for this timing difference may be that β-estradiol was firstly absorbed by roots and then subsequently transported to leaves, meaning a delayed effect on leaves. Nonetheless, most suberin monomers (except for 20:0 primary fatty alcohol) were increased by the 4th day after steroid application in both organs, but this could have occurred a day or two earlier than four days as analysis was not done on those earlier days. Also, accumulation of unpolymerized monomers likely preceded polymer formation, and only the polymer was measured here. The exact sequence of suberin

polymerization is still an open question, but a very detailed time course between zero and four days after steroid application may give insight into the order of monomer accumulation and assembly into oligomers and the suberin polymer.

In Arabidopsis, overexpression of *MYB41* in transgenic Arabidopsis plants using the constitutive 35S promoter leads to ectopic suberin deposition in leaves, but surprisingly there was no increase in root suberin (Kosma *et al*., 2014). According to Kosma *et al*. (2014), two reasons that could account for the lack of root suberin alteration in 35S:*MYB41* transgenic lines were: (1) suberin deposition in roots is already at a maximal level because suberin is mainly synthesized in roots, while leaves are different, as they are starting from almost no suberin; (2) the 35S promoter shows varying activities in different cell types, and for instance the 35S promoter has low activity in seed coats (Young *et al*., 2008). The 35S promoter may also not be highly active in root endodermis, which could account for the lack of suberin induction in 35S:MYB41 lines. In our study, suberin was significantly increased by MYB53 overexpression in both roots and leaves. Therefore, the first possibility where suberin levels are already maxed out in roots is not correct, at least not in young roots. Also, it has been shown that suberin can be produced in root cortex under certain stresses (Barberon *et al*., 2016). The 35S promoter is active in root cortex and therefore the second explanation of why overexpressing AtMYB41 yields no root suberin increases is also likely not correct. An alternative reason is that

AtMYB53 and AtMYB41 regulate suberin deposition by different molecular mechanisms, such as AtMYB53 and AtMYB41 using different co-regulators and/or intermediary regulators for elevating suberin production, which vary between tissues, or that different post-translational modifications (PTMs) act on the two MYBs and these PTMs may also vary between leaves and roots.

4.3 *MYB53, MYB92* and *MYB93* have partially overlapping roles in regulating root endodermal suberin biosynthesis

In the *MYB53/92/93 loss-of-function* lines, suberin is reduced by about 39%-49% in *myb* double mutants compared with wild-type, which is roughly additive reduction levels of the corresponding *myb* single mutants (each single mutant had root suberin reduced by 19%-29%). Similarly, compared with wild-type, suberin reduction was about 72% in the *myb* triple mutants, which is roughly the total of the reductions in the three single *myb53, myb92* and *myb93* mutants. Therefore, *MYB53, MYB92* and *MYB93* have overlapping regulatory functions in root endodermis to govern suberin biosynthesis. This also means that a large proportion of suberin in young roots (2-week-old) of Arabidopsis is dependent on the combined regulatory functions of *MYB53, MYB92* and *MYB93*, at least under normal developmental conditions (non-stress conditions). Meanwhile, *MYB53, MYB92* and *MYB93* may all play other roles in addition

to regulating root suberin, and indeed it has been shown that *myb93* loss-of-function mutants have more lateral roots than wild-type (Gibbs *et al*., 2014). It is possible that suberin deposition and lateral root formation are linked, or it is possible that these MYBs have many functions and may even have very fundamental roles in regulating root cell differentiation, which may then influence multiple aspects of root development, including suberin deposition or lateral root formation. However, it has not been confirmed if these three MYB genes are completely redundant in their functions. It has been reported that the expression patterns are slightly different among the three MYBs. *MYB53* and *MYB92* are expressed in many plant organs and are especially enriched in roots, whereas *MYB93* is seemingly only expressed in roots (Gibbs *et al*., 2014). This implies that MYB93 is restricted to regulatory roles related to roots, while MYB53 and MYB92 may have additional unknown functions.

Even in our triple myb *loss-of-function* mutants, approximately 30% of suberin remains. This indicates that other transcription factors also participate in suberin biosynthesis in young Arabidopsis roots during normal development. Thus, it will be important to investigate the potential roles of other MYB transcription factor members, especially those that are in neighbouring phylogenetic clades. These MYB transcription factors, or even other types of transcription factors, may also contribute to suberin production in young root endodermis under non-stress conditions.

4.4 Implications of MYB-regulated suberin biosynthesis for other plant species

It has been reported that apple *MYB93* (*MdMYB93*), which is a close homolog of Arabidopsis *MYB53*, *MYB92*, and *MYB93*, and a suite of suberin biosynthetic genes are all expressed higher in fruit skins of russeted (suberized) apple varieties compared with fruit skins of waxy (non-suberized) apple varieties (Legay *et al.*, 2015). In a follow up study, it was found that suberin and precursor soluble suberin monomers, along with a collection of predicted suberin biosynthetic genes, are upregulated by transient overexpression of *MdMYB93* in leaves of *Nicotiana benthamiana* (Legay *et al.*, 2016). This supports a role for *MYB53/MYB92/MYB93*-like genes in regulating suberin biosynthesis across diverse plant taxa. Additionally, according to a broad comparison study of suberin deposition in Arabidopsis seeds, several organs of tomato (*Solanum lycopersicum*) including suberized fruit skins, russeted apple (*Malus x domestica*) fruit surfaces, grapevine (*Vitis vinifera*), potato (*Solanum tuberosum*), and roots of rice (*Oryza sativarice*) under waterlogging conditions (Lashbrooke *et al*., 2016), homologs of Arabidopsis *MYB9* and *MYB107* were co-expressed with suberin biosynthetic genes in all these plant species. MYB9 and MYB107 are in a neighbouring phylogenetic clade of MYB53/MYB92/MYB93 when all Arabidopsis MYB protein sequences are compared (Dubos *et al.*, 2010). Therefore, it is possible that these many highly related MYBs

generally regulate suberin biosynthesis in all plants, with individual MYBs having tissue-specific or environmental-specific roles as discussed above.

Since suberin is a hydrophobic barrier that helps plants to withstand stressful environmental conditions such as drought, salt stress, heavy metals or low oxygen, the identification of master regulators of suberin biosynthesis, such as *AtMYB53*, is an important step forward as it provides a means for generating plants with altered cell wall barrier properties (e.g. via altered *MYB53* gene activity). This may provide a novel strategy to generate crops with enhanced stress resistances.

Chapter 5: Future directions and concluding remarks

5.1 Future directions

In this study, I have developed a complete collection of Arabidopsis *MYB53/92/93* single, double and triple *loss-of-function* mutants and developed an Arabidopsis steroid inducible *MYB53* overexpression line. Here, I used these lines to provide evidence that these MYB transcription factors are involved in regulating suberin deposition in an overlapping fashion. In the future, this important collection of plant lines can be used to further understand how suberin production is regulated in roots and the importance of suberin in root physiological functioning. The key outstanding questions to address using these plant tools are:

(1) In Arabidopsis roots, does *MYB53* overexpression cause an increase in suberin deposition specifically in endodermis, where suberin is normally deposited, or also in other root tissues, such as cortex? Also, what leaf tissues are suberized when *MYB53* is overexpressed?

(2) What are the molecular mechanisms by which MYB53/MYB92/MYB93 control suberin deposition? Do they directly or indirectly regulate the expression of suberin biosynthetic genes?

(3) Do these MYB transcription factors function as part of a transcription factor complex and what are their interacting partners?

(4) What are the effects on plant physiology in the *myb53/myb92/myb93* single, double and triple mutants? Are they compromised in water and nutrient relations? Are they more or less susceptible to abiotic and biotic stresses?

I expand upon these four future directions below.

It is known that suberin is variously deposited in a tissue-specific manner during normal development, for example specifically in the exodermis and endodermis of young roots (Schreiber *et al*., 1999; Pollard *et al*., 2008; Schreiber, 2010; Nawrath *et al*., 2013). Under stressful conditions, such as wounding or high salt, suberin production can be induced, including in tissues that are not normally suberized (Vishwanath *et al.*, 2015). For example, treatment of young roots with high NaCl or the stress hormone ABA causes suberin deposition and *GPAT5* expression to expand into the root cortex (Barberon *et al*., 2016). In our biochemical experiments, suberin was greatly induced in both roots and leaves by overexpression of *AtMYB53*. In roots, we currently do not know whether the extra suberin from *MYB53* overexpression is due to more suberin in root endodermis specifically, or from suberin being deposited in additional tissues layers (i.e. the cortex), or both. Also, in leaves, we currently do not know if all cells are producing suberin upon *MYB53* overexpression or only in certain leaf cell types. Microscopic analysis using transmission electron microscopy to examine cell walls for suberin lamallae or using the suberin-specific fluorescent stain fluorol yellow followed by confocal imaging should be

done to identify the tissues that have become suberized upon *MYB53* overexpression.

Although *At*MYB53/92/93 are involved in regulating suberin biosynthesis according to our results, it is unknown whether these MYB transcription factors are directly or indirectly controlling the transcription of downstream suberin-related genes (*CYP86A1*, *CYP86B1*, *GPAT5*, etc). That is, we do not know if AtMYB53/92/93 bind to the promoters of these suberin biosynthetic genes or if they bind to the promotors of genes encoding other transcription factors, which in turn regulate the suberin biosynthetic genes. Arabidopsis MYB107 was recently found to directly target suberin-related genes in seed coats (Gou *et al*., 2017). AtMYB107 is in a closely related subclade to AtMYB53/92/93 when viewed in a phylogenetic tree of all Arabidopsis MYB transcription factors (Dubos *et al.*, 2010). Thus, it is important to determine the direct target genes of AtMYB53/92/93. This can be achieved on a multi-gene or even genome-level scale using Chromatin ImmunoPrecipitation (ChIP)-qPCR or ChIP-seq, respectively. For this, the coding sequence of an epitope tag is fused with the open reading frame of *AtMYB53, MYB92,* or *MYB93* and then put under the control of the 35S promoter or native promoter followed by introduction into wild-type *Arabidopsis thaliana* using Agrobacterium-mediated transformation. Transformed plants are harvested and chromatin chemically cross-linked to fix the protein (MYB transcription factors)-DNA complexes. The extracted chromatin (with proteins cross-linked) is

sonicated to small fragments and then immunoprecipitated with an antibody that binds specifically to the epitope tag to bring down MYB53/92/93-DNA complexes. Later these immune complexes of antibody-MYB-tag bound chromatin are purified and then the eluted chromatin is treated to reversed protein-DNA cross-linking. The eluted pieces of chromatin DNA are further purified to remove remained proteins to obtain the direct target fragments of the MYB transcription factors. These specific DNA fragments can be quantified by qPCR or by next generation sequencing (Kim *et al*., 2013; Fornalé *et al*., 2010). It should be noted that R2R3 MYBs preferentially bind to specific AC-rich sequences (ACC(T/A)ACC or ACCCGCC) (Chezem and Clay, 2016). These results were obtained mainly by *in vitro* promoter binding studies, such as electrophoretic mobility shift assays (EMSA) and / or systematic evolution of ligands by exponential enrichment (SELEX) (Grotewold *et al*., 1994; Zhao *et al.*, 2007; Fornalé *et al*., 2010). Some of these AC-rich target sequences have been confirmed to be true *in vivo* targets using ChIP (Fornalé *et al*., 2010). Inspection of the promoters of Arabidopsis suberin biosynthetic genes reveals AC-rich sequence motifs and the ChIP experiments could target these motifs to test if they are indeed *cis*-regulatory elements that are bound by AtMYB53, MYB92, or MYB93.

It is known that MYB proteins interact with other proteins, such as bHLH and WD40 factors, to form functional transcriptional protein complexes (Ramsay, *et al*.,

2005; Feller *et al*., 2011). It has already been reported that AtMYB53/92/93 share a conserved amino acid sequence C-terminal of the R2R3 MYB DNA-binding domain that is not found in any other Arabidopsis MYB proteins (Stracke *et al*., 2001; Dubos *et al*., 2010; Gibbs *et al*., 2014). This consensus amino acid motif is necessary for MYB93 to interact with ARABIDILLO, which is a positive regulator of lateral root initiation (Gibbs *et al*., 2014). According to yeast-two-hybrid results, AtMYB53/92/93 all interacted with the ARABIDILLO ARMADILLO (ARM) domain, which is hypothesized to mediate protein-protein interactions. It is likely that AtMYB53/92/93 additionally interacts with other co-factors in the context of regulated suberin biosynthesis. Additional research could be further conducted about the protein-protein interactions involving AtMYB53/92/93 by using yeast-two-hybrid screening or co-immunoprecipitation coupled with mass spectrometry.

Finally, the whole collection of *myb53/92/93* single, double and triple *loss-of-function* mutants also can be used to conduct a series of phenotypic experiments to further determine the protective functions of suberin in plant physiology. These mutants could be tested for altered stress resistance against extreme environmental conditions such as drought or extreme heat, and soil with high salt or toxic metals. Altered resistance/susceptibility to root pathogens could also be examined in these mutants compared to wild-type.

5.2 Concluding remarks

In conclusion, the findings from our study indicate that *AtMYB53/92/93* have overlapping functions in positively regulating suberin biosynthesis in roots during normal development. Therefore, these transcription factors can be used to coordinately upregulate suberin biosynthetic genes and to manipulate the deposition of suberin in Arabidopsis.

Suberin serves as a hydrophobic barrier for plant protection by controlling water, gas and solute movement, as well as restricting pathogen attack. There is much evidence indicating that suberized tissues help plants to withstand unfavorable environmental conditions such as drought, chill, salt stress, heavy metals, and anoxia (Lulai and Corsini, 1998; Enstone *et al*., 2003; Franke and Schreiber 2007; Schreiber 2010). A better understanding of the molecular mechanisms governing the regulated deposition of suberin may be used to develop stress tolerant crops. In addition, suberin consists of oxygenated fatty acids and these components can be used in a wide range of industrial products including resins, coatings, dyes, nylons, plastics, soaps, inks and biofuel (Li and Beisson, 2009). It is thus promising that suberin-enriched plants can be developed to produce high value bio-lipids with low environmental costs.

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Appendix A.

Lipid polyester (suberin/cutin) monomer composition (absolute values) of *MYB53* inducible overexpression line treated with β-estradiol or DMSO only (mock control) seedlings over a timecourse. Data are represented as average value ± SE (µg monomer per mg of delipidated dry residue) in roots (Table S1) or leaves (Table S2), n=3 replicates.

Table S1. Suberin monomer composition in roots of β-estradiol treated seedlings relative to the corresponding mock control seedlings (DMSO only).

The red-coloured data represent statistical significance compared pair-wise between mock control and treated groups (P<0.05, LSD comparison).

Suberin Components	Average Value of Suberin Components and Total Suberin in Roots Tissue (Dry Weight µg/mg)							
	Mock	1 day treatment	Mock	4 days treatment	Mock	7 days treatment	Mock	10 days treatment
	(11 days growth)		(14 days growth)		(17 days growth)		(20 days growth)	
Hydroxycinnamic Acid Methyl Esters								
Ferulate	0.28 ± 0.02	0.38 ± 0.01	0.31 ± 0.02	1.77 ± 0.06	0.15 ± 0.01	0.65 ± 0.02	0.19 ± 0.01	0.73 ± 0.07
Fatty Acid Methyl Esters								
C20:0	0.45 ± 0.03	0.50 ± 0.04	0.55 ± 0.00	2.65 ± 0.02	0.57 ± 0.04	2.26 ± 0.03	0.40 ± 0.00	1.25 ± 0.05
C22:0	1.87 ± 0.10	2.17 ± 0.17	2.35 ± 0.01	9.58 ± 0.09	2.26 ± 0.15	8.55 ± 0.25	1.75 ± 0.02	5.02 ± 0.16
C24:0	0.63 ± 0.03	0.79 ± 0.06	0.76 ± 0.01	4.22 ± 0.06	0.70 ± 0.05	4.18 ± 0.12	0.57 ± 0.01	2.55 ± 0.14
Dicarboxylic Fatty Acid Dimethyl Esters								
C16:0	1.27 ± 0.07	1.42 ± 0.10	1.49 ± 0.02	6.63 ± 0.03	1.38 ± 0.10	5.28 ± 0.13	1.06 ± 0.00	3.07 ± 0.12
C18:2	0.58 ± 0.03	0.69 ± 0.07	0.68 ± 0.00	2.43 ± 0.17	0.49 ± 0.06	1.68 ± 0.11	0.44 ± 0.01	1.17 ± 0.03
C18:1	3.91 ± 0.25	4.52 ± 0.33	4.58 ± 0.07	21.07 ± 0.09	4.28 ± 0.32	18.79 ± 0.53	3.41 ± 0.04	10.40 ± 0.37
C18:0	0.63 ± 0.04	0.68 ± 0.06	0.68 ± 0.01	3.01 ± 0.04	0.78 ± 0.06	2.92 ± 0.05	0.44 ± 0.00	1.45 ± 0.06
C20:0	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.00	0.71 ± 0.01	0.19 ± 0.02	0.68 ± 0.02	0.10 ± 0.00	0.33 ± 0.03
C22:0	0.21 ± 0.01	0.33 ± 0.02	0.24 ± 0.00	1.86 ± 0.03	0.29 ± 0.02	1.89 ± 0.07	0.18 ± 0.00	1.16 ± 0.21
ω-Hydroxyl Fatty Acid Methyl Esters								
C16:0	0.53 ± 0.03	0.65 ± 0.05	0.64 ± 0.01	3.63 ± 0.04	0.51 ± 0.03	2.58 ± 0.04	0.46 ± 0.00	1.75 ± 0.07
C18:2	0.31 ± 0.02	0.39 ± 0.03	0.41 ± 0.01	1.81 ± 0.03	0.29 ± 0.02	1.11 ± 0.03	0.27 ± 0.01	0.77 ± 0.03
C18:1	4.01 ± 0.26	4.77 ± 0.35	4.83 ± 0.15	24.15 ± 0.44	4.25 ± 0.30	19.62 ± 0.31	3.54 ± 0.06	11.88 ± 0.52
C18:0	0.19 ± 0.01	0.21 ± 0.02	0.20 ± 0.00	0.84 ± 0.03	0.30 ± 0.02	0.82 ± 0.01	0.14 ± 0.00	0.43 ± 0.03
C20:0	0.29 ± 0.02	0.33 ± 0.03	0.31 ± 0.00	1.34 ± 0.02	0.38 ± 0.03	1.27 ± 0.02	0.23 ± 0.00	0.77 ± 0.07
C22:0	1.01 ± 0.05	1.41 ± 0.11	1.21 ± 0.03	6.14 ± 0.02	1.03 ± 0.07	4.98 ± 0.12	0.93 ± 0.02	3.32 ± 0.15
C24:0	0.27 ± 0.01	0.26 ± 0.13	0.33 ± 0.01	1.73 ± 0.02	0.25 ± 0.02	1.39 ± 0.03	0.24 ± 0.01	1.07 ± 0.04
Primary Fatty Alcohols								
C18:0	0.30 ± 0.02	0.31 ± 0.03	0.22 ± 0.00	0.83 ± 0.01	0.20 ± 0.02	0.82 ± 0.02	0.18 ± 0.00	0.68 ± 0.02
C20:0	0.14 ± 0.01	0.13 ± 0.03	0.15 ± 0.00	0.26 ± 0.09	0.13 ± 0.00	0.42 ± 0.05	0.12 ± 0.00	0.37 ± 0.01
C22:0	0.12 ± 0.01	0.13 ± 0.01	0.11 ± 0.00	0.33 ± 0.01	0.08 ± 0.01	0.28 ± 0.01	0.10 ± 0.00	0.31 ± 0.02
Total	17.73 ± 1.02	20.73 ± 1.39	20.57 ± 0.37	95.36 ± 0.64	18.68 ± 1.32	80.44 ± 1.73	15.09 ± 0.23	48.83 ± 2.17

Table S2. Lipid polyester (cutin + suberin) monomer composition in leaves of β-estradiol treated seedlings relative to the corresponding mock control seedlings (DMSO only).

The red-coloured data represent statistical significance compared pair-wise between mock control and treated groups (P<0.05, LSD comparison).

Lipid Polyester Components (Suberin/Cutin)	Average Value of Polyester components and Total Polyester in Leaves Tissue (Dry Weight µg/mg)							
	Mock	1 day treatment	Mock	4 days treatment	Mock	7 days treatment	Mock	10 days treatment
	(11 days growth)		(14 days growth)		(17 days growth)		(20 days growth)	
Hydroxycinnamic Acid Methyl Esters								
Ferulate	0.07 ± 0.01	0.07 ± 0.00	0.08 ± 0.01	0.19 ± 0.03	0.01 ± 0.00	0.13 ± 0.00	0.07 ± 0.00	0.14 ± 0.01
Fatty Acid Methyl Esters								
C20:0	0.04 ± 0.01	0.04 ± 0.00	0.05 ± 0.00	0.13 ± 0.02	0.08 ± 0.00	0.19 ± 0.00	0.04 ± 0.00	0.13 ± 0.01
C22:0	0.10 ± 0.02	0.12 ± 0.01	0.13 ± 0.01	0.40 ± 0.06	0.16 ± 0.01	0.62 ± 0.01	0.09 ± 0.00	0.39 ± 0.02
C24:0	0.05 ± 0.01	0.05 ± 0.00	0.07 ± 0.01	0.14 ± 0.02	0.09 ± 0.00	0.22 ± 0.00	0.04 ± 0.00	0.12 ± 0.01
Dicarboxylic Fatty Acid Dimethyl Esters								
C16:0	0.22 ± 0.03	0.22 ± 0.02	0.24 ± 0.02	0.47 ± 0.06	0.34 ± 0.01	0.57 ± 0.01	0.21 ± 0.01	0.42 ± 0.04
C18:2	1.53 ± 0.25	1.48 ± 0.10	1.32 ± 0.21	1.66 ± 0.13	1.72 ± 0.11	1.71 ± 0.06	0.84 ± 0.10	0.95 ± 0.09
C18:1	0.35 ± 0.05	0.37 ± 0.02	0.38 ± 0.04	0.99 ± 0.15	0.53 ± 0.02	1.40 ± 0.04	0.25 ± 0.01	0.92 ± 0.11
C18:0	0.10 ± 0.02	0.10 ± 0.00	0.12 ± 0.01	0.19 ± 0.01	0.17 ± 0.01	0.27 ± 0.00	0.09 ± 0.02	0.19 ± 0.02
C20:0	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.08 ± 0.00	0.02 ± 0.00	0.05 ± 0.00
C22:0	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.11 ± 0.02	0.04 ± 0.01	0.21 ± 0.00	0.03 ± 0.00	0.10 ± 0.01
ω-Hydroxyl Fatty Acid Methyl Esters								
C16:0	0.05 ± 0.01	0.05 ± 0.00	0.06 ± 0.00	0.18 ± 0.03	0.08 ± 0.00	0.21 ± 0.01	0.05 ± 0.00	0.16 ± 0.01
C18:2	0.11 ± 0.02	0.11 ± 0.00	0.11 ± 0.02	0.19 ± 0.02	0.11 ± 0.01	0.18 ± 0.00	0.07 ± 0.01	0.13 ± 0.01
C18:1	0.23 ± 0.04	0.26 ± 0.03	0.31 ± 0.02	0.99 ± 0.16	0.45 ± 0.02	1.42 ± 0.05	0.22 ± 0.00	0.94 ± 0.08
C18:0	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.11 ± 0.00	0.02 ± 0.00	0.05 ± 0.00
C20:0	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.10 ± 0.01	0.08 ± 0.00	0.16 ± 0.00	0.02 ± 0.00	0.08 ± 0.01
C22:0	0.08 ± 0.02	0.09 ± 0.01	0.10 ± 0.01	0.33 ± 0.05	0.11 ± 0.01	0.48 ± 0.01	0.06 ± 0.00	0.29 ± 0.02
C24:0	0.05 ± 0.01	0.03 ± 0.00	0.04 ± 0.00	0.07 ± 0.01	0.03 ± 0.00	0.10 ± 0.00	0.02 ± 0.00	0.06 ± 0.00
Primary Fatty Alcohols								
C18:0	0.05 ± 0.01	0.05 ± 0.01	0.02 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.07 ± 0.00	0.02 ± 0.00	0.06 ± 0.00
C20:0	0.23 ± 0.01	0.29 ± 0.02	0.21 ± 0.02	0.22 ± 0.02	0.11 ± 0.01	0.11 ± 0.01	0.31 ± 0.02	0.29 ± 0.01
C22:0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.04 ± 0.00
Total	3.85 ± 0.54	3.87 ± 0.14	4.02 ± 0.46	7.16 ± 0.81	5.00 ± 0.25	9.04 ± 0.15	3.33 ± 0.19	6.30 ± 0.47

Appendix B.

Suberin monomer composition (absolute values) of wild-type and *MYB53/MYB92/MYB93* single, double and triple mutant lines grown in 4 batches (Tables S3-S6). Data are represented as average value±SE in roots, n=3 replicates.

Table S3. Suberin monomer composition in wild-type, *myb53-1, myb53-1 myb92-1* and *myb53-1 myb93-1*.

Suberin Components	Average Value of Suberin Components and Total Suberin (µg/mg Dry Weight)			
	Wild Type	*myb53-1*	*myb53-1 myb92-1*	*myb53-1 myb93-1*
Hydroxycinnamic Acid Methyl Esters				
Ferulate	0.20 ± 0.02	0.17 ± 0.02	0.15 ± 0.03	0.14 ± 0.03
Fatty Acid Methyl Esters				
C16:0	0.31 ± 0.05	0.22 ± 0.02	0.38 ± 0.02	0.37 ± 0.07
C18:0	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01
C20:0	0.27 ± 0.03	0.24 ± 0.02	0.18 ± 0.04	0.21 ± 0.05
C22:0	1.03 ± 0.07	0.93 ± 0.01	0.48 ± 0.08	0.55 ± 0.04
C24:0	0.22 ± 0.02	0.18 ± 0.01	0.15 ± 0.03	0.17 ± 0.04
Dicarboxylic Fatty Acid Dimethyl Esters				
C16:0	0.83 ± 0.07	0.75 ± 0.03	0.46 ± 0.04	0.53 ± 0.04
C18:2	0.29 ± 0.08	0.24 ± 0.00	0.14 ± 0.02	0.17 ± 0.02
C18:1	1.81 ± 0.12	1.12 ± 0.06	0.78 ± 0.06	0.89 ± 0.09
C18:0	0.40 ± 0.04	0.34 ± 0.03	0.23 ± 0.03	0.26 ± 0.07
C20:0	0.11 ± 0.01	0.09 ± 0.01	0.06 ± 0.01	0.07 ± 0.02
C22:0	0.13 ± 0.01	0.11 ± 0.01	0.08 ± 0.01	0.10 ± 0.02
ω-Hydroxyl Fatty Acid Methyl Esters				
C16:0	0.44 ± 0.05	0.37 ± 0.02	0.27 ± 0.05	0.32 ± 0.08
C18:2	0.25 ± 0.03	0.20 ± 0.00	0.12 ± 0.02	0.15 ± 0.02
C18:1	2.48 ± 0.25	1.95 ± 0.06	1.06 ± 0.04	1.25 ± 0.04
C18:0	0.13 ± 0.02	0.10 ± 0.01	0.09 ± 0.01	0.10 ± 0.03
C20:0	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.01
C22:0	0.79 ± 0.07	0.64 ± 0.03	0.47 ± 0.07	0.56 ± 0.13
C24:0	0.16 ± 0.01	0.11 ± 0.00	0.06 ± 0.01	0.07 ± 0.02
Primary Fatty Alcohols				
C18:0	0.25 ± 0.03	0.25 ± 0.02	0.18 ± 0.03	0.21 ± 0.07
C20:0	0.17 ± 0.04	0.16 ± 0.01	0.09 ± 0.03	0.11 ± 0.01
C22:0	0.15 ± 0.02	0.15 ± 0.01	0.06 ± 0.01	0.07 ± 0.03
Total Suberin Content	10.44 ± 0.91	8.43 ± 0.18	5.53 ± 0.66	6.34 ± 0.75

Table S4. Suberin monomer composition in wild-type, *myb53-2 myb92-1, myb53-2 myb93-1* and *myb53-2 myb92-1 myb93-1.*

Suberin Components	Average Value of Suberin Components and Total Suberin (µg/mg Dry Weight)			
	Wild Type	*myb53-2 myb92-1*	*myb53-2 myb93-1*	*myb53-2 myb92-1 myb93-1*
Hydroxycinnamic Acid Methyl Esters				
Ferulate	0.19 ± 0.04	0.14 ± 0.03	0.12 ± 0.01	0.12 ± 0.01
Fatty Acid Methyl Esters				
C16:0	0.30 ± 0.04	0.19 ± 0.04	0.26 ± 0.05	0.11 ± 0.02
C18:0	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.00 ± 0.00
C20:0	0.28 ± 0.06	0.20 ± 0.00	0.21 ± 0.04	0.10 ± 0.02
C22:0	1.06 ± 0.20	0.52 ± 0.07	0.56 ± 0.06	0.32 ± 0.04
C24:0	0.22 ± 0.04	0.16 ± 0.02	0.18 ± 0.02	0.11 ± 0.03
Dicarboxylic Fatty Acid Dimethyl Esters				
C16:0	0.85 ± 0.17	0.48 ± 0.02	0.54 ± 0.02	0.28 ± 0.02
C18:2	0.30 ± 0.02	0.15 ± 0.01	0.17 ± 0.05	0.08 ± 0.03
C18:1	1.85 ± 0.36	0.81 ± 0.07	0.90 ± 0.03	0.26 ± 0.03
C18:0	0.41 ± 0.08	0.24 ± 0.02	0.26 ± 0.05	0.13 ± 0.03
C20:0	0.11 ± 0.02	0.06 ± 0.01	0.07 ± 0.01	0.04 ± 0.01
C22:0	0.13 ± 0.02	0.09 ± 0.02	0.10 ± 0.01	0.08 ± 0.01
ω-Hydroxyl Fatty Acid Methyl Esters				
C16:0	0.45 ± 0.11	0.28 ± 0.01	0.33 ± 0.05	0.15 ± 0.04
C18:2	0.26 ± 0.02	0.13 ± 0.00	0.15 ± 0.00	0.06 ± 0.01
C18:1	2.54 ± 0.15	1.10 ± 0.03	1.27 ± 0.05	0.49 ± 0.03
C18:0	0.13 ± 0.01	0.09 ± 0.00	0.10 ± 0.02	0.05 ± 0.01
C20:0	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
C22:0	0.81 ± 0.02	0.47 ± 0.06	0.58 ± 0.06	0.32 ± 0.06
C24:0	0.16 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.05 ± 0.01
Primary Fatty Alcohols				
C18:0	0.26 ± 0.02	0.18 ± 0.01	0.22 ± 0.03	0.17 ± 0.06
C20:0	0.17 ± 0.03	0.09 ± 0.00	0.11 ± 0.02	0.06 ± 0.04
C22:0	0.16 ± 0.01	0.06 ± 0.00	0.07 ± 0.01	0.05 ± 0.01
Total Suberin Content	10.70 ± 1.27	5.52 ± 0.23	6.33 ± 0.55	3.04 ± 0.45

Table S5. Suberin monomer composition in wild-type, *myb53-2, myb92-1* and *myb93-1*.

Suberin Components	Average Value of Suberin Components and Total Suberin (µg/mg Dry Weight)			
	Wild Type	*myb53-2*	*myb92-1*	*myb93-1*
Hydroxycinnamic Acid Methyl Esters				
Ferulate	0.18 ± 0.02	0.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.00
Fatty Acid Methyl Esters				
C16:0	0.20 ± 0.05	0.25 ± 0.10	0.22 ± 0.04	0.13 ± 0.03
C18:0	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C20:0	0.29 ± 0.04	0.23 ± 0.02	0.23 ± 0.02	0.19 ± 0.02
C22:0	1.15 ± 0.04	0.82 ± 0.13	0.85 ± 0.02	0.92 ± 0.14
C24:0	0.23 ± 0.03	0.19 ± 0.02	0.18 ± 0.03	0.15 ± 0.02
Dicarboxylic Fatty Acid Dimethyl Esters				
C16:0	0.87 ± 0.11	0.71 ± 0.06	0.61 ± 0.06	0.62 ± 0.08
C18:2	0.31 ± 0.02	0.25 ± 0.04	0.18 ± 0.01	0.24 ± 0.03
C18:1	1.93 ± 0.10	1.43 ± 0.11	1.20 ± 0.01	1.43 ± 0.07
C18:0	0.44 ± 0.06	0.33 ± 0.04	0.33 ± 0.05	0.26 ± 0.01
C20:0	0.12 ± 0.02	0.08 ± 0.01	0.10 ± 0.02	0.06 ± 0.01
C22:0	0.15 ± 0.02	0.12 ± 0.01	0.11 ± 0.02	0.15 ± 0.02
ω-Hydroxyl Fatty Acid Methyl Esters				
C16:0	0.50 ± 0.05	0.36 ± 0.03	0.38 ± 0.02	0.35 ± 0.04
C18:2	0.28 ± 0.03	0.19 ± 0.00	0.20 ± 0.01	0.20 ± 0.02
C18:1	2.69 ± 0.11	1.91 ± 0.14	1.73 ± 0.07	1.83 ± 0.06
C18:0	0.14 ± 0.02	0.10 ± 0.01	0.11 ± 0.01	0.08 ± 0.00
C20:0	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C22:0	0.86 ± 0.09	0.70 ± 0.06	0.74 ± 0.12	0.85 ± 0.09
C24:0	0.17 ± 0.02	0.13 ± 0.02	0.11 ± 0.01	0.17 ± 0.02
Primary Fatty Alcohols				
C18:0	0.29 ± 0.03	0.28 ± 0.02	0.23 ± 0.01	0.17 ± 0.02
C20:0	0.19 ± 0.03	0.18 ± 0.03	0.14 ± 0.01	0.13 ± 0.01
C22:0	0.16 ± 0.01	0.15 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
Total Suberin Content	11.20 ± 0.67	8.62 ± 0.62	7.94 ± 0.33	8.22 ± 0.60

Table S6. Suberin monomer composition in wild-type, *myb92-1 myb93-1* and *myb53-1 myb92-1 myb93-1*.

Suberin Components	Average Value of Suberin Components and Total Suberin (µg/mg Dry Weight)		
	Wild Type	*myb92-1 myb93-1*	*myb53-1 myb92-1 myb93-1*
Hydroxycinnamic Acid Methyl Esters			
Ferulate	0.25 ± 0.06	0.16 ± 0.01	0.12 ± 0.02
Fatty Acid Methyl Esters			
C16:0	0.30 ± 0.10	0.28 ± 0.04	0.42 ± 0.07
C18:0	0.03 ± 0.01	0.02 ± 0.00	0.04 ± 0.02
C20:0	0.34 ± 0.07	0.16 ± 0.01	0.10 ± 0.02
C22:0	1.51 ± 0.23	0.54 ± 0.05	0.33 ± 0.06
C24:0	0.45 ± 0.07	0.15 ± 0.01	0.11 ± 0.02
Dicarboxylic Fatty Acid Dimethyl Esters			
C16:0	1.02 ± 0.19	0.45 ± 0.03	0.29 ± 0.06
C18:2	0.30 ± 0.06	0.12 ± 0.01	0.08 ± 0.01
C18:1	1.44 ± 0.35	0.51 ± 0.04	0.27 ± 0.08
C18:0	0.48 ± 0.11	0.21 ± 0.02	0.13 ± 0.03
C20:0	0.14 ± 0.03	0.07 ± 0.01	0.04 ± 0.01
C22:0	0.23 ± 0.04	0.12 ± 0.01	0.08 ± 0.02
ω-Hydroxyl Fatty Acid Methyl Esters			
C16:0	0.44 ± 0.09	0.23 ± 0.02	0.16 ± 0.04
C18:2	0.27 ± 0.06	0.10 ± 0.01	0.06 ± 0.01
C18:1	2.84 ± 0.50	0.97 ± 0.08	0.51 ± 0.11
C18:0	0.14 ± 0.03	0.07 ± 7.72	0.05 ± 0.01
C20:0	0.31 ± 0.06	0.12 ± 0.01	0.09 ± 0.02
C22:0	1.06 ± 0.14	0.55 ± 0.06	0.33 ± 0.06
C24:0	0.27 ± 0.03	0.10 ± 0.01	0.06 ± 0.01
Primary Fatty Alcohols			
C18:0	0.31 ± 0.05	0.22 ± 0.01	0.18 ± 0.04
C20:0	0.21 ± 0.03	0.10 ± 0.01	0.06 ± 0.01
C22:0	0.21 ± 0.02	0.07 ± 0.01	0.05 ± 0.01
Total Suberin Content	12.56 ± 2.29	5.34 ± 0.37	3.57 ± 0.70

Appendix C.

Table S7. Summary of the p-values calculated by statistical analysis (p<0.05, LSD multiple comparison) between the content of each suberin monomer and also total suberin in the *myb53-1* collection of mutants (*myb53-1, myb53-1 myb92-1, myb53-1 myb93-1 and myb53-1 myb92-1 myb93-1*) in comparison with the *myb53-2* collection of mutants (*myb53-2, myb53-2 myb92-1, myb53-2 myb93-1 and myb53-2 myb92-1 myb93-1*).

Suberin Components	Compared To *myb53-2* *myb53-1*	Compared To *myb53-2 myb92-1* *myb53-1 myb92-1*	Compared To *myb53-2 myb93-1* *myb53-1 myb93-1*	Compared To *myb53-2 myb92-1 myb93-1* *myb53-1 myb92-1 myb93-1*
Hydroxycinnamic Acid Methyl Esters				
Ferulate	0.188	0.065	0.685	0.368
Fatty Acid Methyl Esters				
C16:0	0.093	0.050	0.259	0.001
C18:0	0.470	0.895	0.693	0.010
C20:0	0.549	0.812	0.975	0.703
C22:0	0.054	0.808	0.969	0.370
C24:0	0.968	0.969	0.947	0.153
Dicarboxylic Fatty Acid Dimethyl Esters				
C16:0	0.229	0.934	0.921	0.545
C18:2	0.831	0.952	0.968	0.932
C18:1	0.332	0.890	0.927	0.390
C18:0	0.485	0.944	0.958	0.776
C20:0	0.598	0.951	0.958	0.718
C22:0	0.610	0.945	0.961	0.131
ω-Hydroxyl Fatty Acid Methyl Esters				
C16:0	0.341	0.971	0.938	0.865
C18:2	0.136	0.949	0.907	0.941
C18:1	0.058	0.935	0.866	0.708
C18:0	0.629	0.960	0.948	0.998
C20:0	0.825	0.937	0.986	0.708
C22:0	0.998	0.877	0.993	0.553
C24:0	0.983	0.910	0.991	0.209
Primary Fatty Alcohols				
C18:0	1.000	0.914	0.999	0.646
C20:0	0.972	0.473	0.993	0.732
C22:0	0.922	0.925	0.997	0.493
Total Suberin	0.699	0.782	0.815	0.993

Appendix D.

A summary of the primers used in PCR-based experiments.

Table S8. Summary of the primers used for semi-quantitative RT-PCR

Gene	AGI	Primer name (Sequence 5' to 3')
MYB53	At5g65230	MYB53-F1: TGCAAGCCACTTACCTGGAC MYB53-R1: TGGTTCAAGCTCGGTGGTTC
MYB53	At5g65230	MYB53-F2: CCGAGCTTGAACCAAACTATGT MYB53-R2: TTGAGGAGGCTTGGTTGCTG
MYB92	At5g10280	MYB92-F: CCTTCCCAAACTCGCTGGTC MYB92-R: GGATGGATCCGAGAGCCAGA
MYB93	At1g34670	MYB93-F: AAGGGCCATGGACTCCTGAA MYB93-R: AAACTTGCGAAGAGGTCGGT
GAPDH	At1g13440	GAPDH-F: TTGGTGACAACAG^GTCAAGCA GAPDH-R: AAACTTGTCGCTCAATGCAATC

Table S9. Summary of the primers used for quantitative RT-PCR

Gene	AGI	Primer name (Sequence 5' to 3')
FAR1	At5g22500	FAR1-F: ACAGCTCATTCGGGAGACAC FAR1-R: GAGCCGTGAAATCGTGAAGT
FAR4	At3g44540	FAR4-F: AGTCCTTGATCTTATACCTGTGG FAR4-R: GCTTCCCTGCGTGTATTGC
FAR5	At3g44550	FAR5-F: TGTTTGATTTCGACCCAAAAGG FAR5-R: CTTCTTAAGCACGTGTGTGACG
GPAT5	At3g11430	GPAT5-F: TGAGGGAACCACTTGTCGTG GPAT5-R: ATCGCAACCGGAACAATCCT
ASFT	At5g41040	ASFT-F: AACTCATGGGGTCAAGTCGC ASFT-R: CTTTGGAGGGTTTCGAGCATTGAG
CYP86B1/RALPH	At5g23190	RALPH-F: ATCCAGGATGTCTCGGTCCA RALPH-R: TGACGAATCTCACAACCGCA
CYP86A1/HORST	At5g58860	HORST-F: CGCTGCGTTTATACCCTTCTGTGC HORST-R: CTTGGCACGAAAGTCCCGTC
GAPDH	At1g13440	GAPDH-F: TTGGTGACAACAGGTCAAGCA GAPDH-R: AAACTTGTCGCTCAATGCAATC
PP2A	At1g13320	PP2A-F: TAACGTGGCCAAAATGATGC PP2A-R: GTTCTCCACAACCGCTTGGT

Appendix E.

Figure S1. Schematic representation of the phylogenetic relationships in the R2R3-MYB subfamily with members regulating suberin biosynthesis highlighted.

Figure is modified from Dubos *et al*., 2010.