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# Partial characterization of putative CYP86A genes from soybean

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# Abstract

Soybean (*Glycine max* [L.] Merr.) is a globally cultivated crop that is important to the sustainability of many industries. However, like all plants, optimal cultivation of soybean is threatened by detrimental environmental factors. For example, high yield of soybean is threatened by soil-borne pathogens like *Phytophthora sojae*. Resistance against *P. sojae* was previously positively correlated with aliphatic suberin deposition in soybean. As such, a deeper understanding of the biosynthesis of suberin may assist in engineering a resistant form of soybean, based on enhanced suberin content. In soybean, the ω-OH fatty acid monomers are predominant and most strongly correlated with resistance. These ω-OH fatty acids are synthesized by CYP86As (a subfamily of fatty-acid ω-hydroxylase (FAωH) enzymes). In soybean, two putative FAωH genes, *CYP86A37* and *CYP86A38* are expressed in roots. Using a hairy root model system, RNAi knockdown lines for *CYP86A37* and *CYP86A38* were generated. Expression of *CYP86A37* and *CYP86A38* confirmed knockdown of the two genes, and downstream changes in suberin deposition were quantified using GC/MS. Expression of *CYP86A37* and *CYP86A38* was also compared across cultivars (Williams, Conrad, and OX760-6) and a developmental axis, and related to aliphatic suberin deposition. Expression of these two genes positively correlated only with the ω-hydroxylated suberin monomers, particularly 18-hydroxy-oleic acid. Unexpectantly, gene expression and amount of suberin deposition did not relate to known disease resistance in the three cultivars. Overall, my data indicates that CYP86A37 and CYP86A38 likely display fatty-acid ω-hydroxylase function and are therefore likely involved in suberin biosynthesis. As the expression of these two genes impacts the composition of the suberin polymer, it will be important to further explore these genes including developmental regulation to gain insight into the factors contributing to the suberin phenotype.



# Keywords

aliphatic suberin, *CYP86A37*, *CYP86A38*, FAωHs, development, plant-environment interactions

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# List of Abbreviations

| | |
|---|---|
| **4CL** | 4-coumaroyl-CoA ligase |
| **ABCG** | G-type ATP-binding cassette |
| **ABRC** | Arabidopsis Biological Resource Center |
| **ACT-II** | actin 2 |
| **ASFT** | aliphatic suberin feruloyl transferase |
| **C4H** | cinnamate 4-hydroxylase |
| **CAD** | coniferyl alcohol dehydrogenase |
| **CCR** | cinnamoyl-CoA-oxidoreductase |
| **cDNA** | complimentary deoxyribonucleic acid |
| **CFI** | chlorophyll fluorescence imaging |
| **CYP** | cytochrome P450 |
| **DCA** | dicarboxylic acid |
| **ER** | endoplasmic reticulum |
| **ETI** | effector triggered immunity |
| **FACT** | fatty alcohol: caffeoyl-CoA caffeoyl transferase |
| **FAR** | fatty acyl reductase |
| **FAωH** | fatty acid ω-hydroxylase |
| **GC-FID** | gas chromatography with flame ionization detection |
| **GC-MS** | gas chromatography with mass spectrometry |
| **GPAT** | glycerol 3-phosphate acyltransferase |
| **HR** | hypersensitive response |
| **IPCC** | Intergovernmental Panel on Climate Change |
| **JA** | jasmonic acid |
| **KCS** | β-ketoacyl-CoA synthase |
| **LACS** | long-chain acyl-CoA synthase |
| **M-S** | Murashige-Skoog |
| **PAL** | phenylalanine ammonia lyase |
| **PAMP** | pathogen-associated recognition patterns |
| **PCR** | polymerase chain reaction |
| **PR** | pathogenesis-related |
| **PRR** | PAMP recognition receptor |

| | |
|---|---|
| **PTI** | PAMP triggered immunity |
| **qPCR** | quantitative PCR |
| **RNA** | ribonucleic acid |
| **RNAi** | RNA interference |
| **ROS** | reactive oxygen species |
| **RT-PCR** | reverse transcriptase PCR |
| **SA** | salicylic acid |
| **SAR** | systemic acquired resistance |
| **TEM** | transmission election microscopy |
| **TF** | transcription factor |
| **TMS** | trimethylsilyl |
| **VLCFA** | very long chain fatty acid |
| **ω-OH** | ω-hydroxylated fatty acid |

# 1 INTRODUCTION

## 1.1 Understanding plant-environment interaction is key to crop improvement

The adverse environmental conditions plants experience directly impact plant physiology, and over evolutionary time plants have evolved many strategies to maintain homeostasis under conditions of environmental stress, and thereby enhance their ability to survive dynamic environments. Further complicating the already challenging environmental conditions faced by plants are more broad-scale factors like climate change, which is predicted to alter both the abiotic and biotic environment of several large-scale ecosystems over the coming decades (IPCC, 2014). As such, understanding how plants interact with their environment will play a key role in determining not only the role of plants in combatting climate change, but also in informing breeding of crop plants that will persist in the future environment. One way to approach a deeper understanding of how plants interact with their environment is to explore the many strategies plants have developed to cope with challenging environmental factors.

For the agricultural industry, developing cultivars that are resistant to environmental stressors is of great interest, and generally requires analysis of response to specific stressors. Abiotically, the three most agronomically important sources of stress are drought, high salinity, and extreme temperatures (Fraire-Velázquez et al. 2011; Gupta et al. 2014; Fang and Xiong 2015; Parihar et al. 2015). Together with diseases, which account for 14.1% of crop losses worldwide (Agrios 2005b), these key stressors can account for a large proportions of annual crop losses. To prevent such losses in the future will require the pursuit of cultivars that are highly resistant to these key stressors. However, in this pursuit it is important to first gain a better understanding of innate defense mechanisms prior to attempting to enhance specific mechanistic features in specific crop plants. Without this initial understanding, we lack the knowledge required to enhance these innate stress response mechanisms in already established cultivars.

One strategy plants have evolved for combating adverse environmental conditions, is the biosynthesis of preformed barriers like suberin. As suberin has been shown to aid in both defense against pathogens and coping with the main agronomically relevant abiotic

stressors, it is of particular interest in developing highly resistant cultivars. Consequently, I have approached this thesis from the perspective that the preformed barrier suberin is a key aspect of defense against adverse environmental conditions. As pathogen attack contributes an 11% economic loss annually for the soybean (*Glycine max*) agriculture industry (Ramachandra et al. 2015), it is not unreasonable to consider development of soybean cultivars with enhanced suberin deposition, given suberin plays a role in resistance against pathogen attack. Of particular interest to soybean producers is the plant-pathogen interaction between soybean and the root rot causing pathogen *Phytophthora sojae* (Kaufmann & Gerdemann). *Phytophthora sojae* is the 4th most scientifically and economically important plant pathogenic oomycete (Kamoun et al. 2015). Due to the high host specificity of *P. sojae* to soybean (Tyler 2002), infection by *P. sojae* occurs in almost all areas where soybean is grown and accounts for approximately 1-2 billion dollars (USD) in global losses per year (Tyler 2007). Therefore, soybean cultivars that are highly resistant against *P. sojae* are likely to play a key role in reducing future yield losses.

In this thesis, I emphasize gaining a better understanding of the biosynthesis suberin aliphatics, as they are thought to be more integral to the suberin-resistance phenomenon regarding pathogen attack by *P. sojae* (Thomas et al. 2007). To enhance the understanding of the role of suberin in plant defense, I have explored key suberin biosynthetic genes and their expression in cultivars with different field-level tolerance to *P. sojae* infection. I have also considered different developmental ages of tissue, as suberin is known to be deposited in distinct developmental stages and *P. sojae* is more likely to attack younger tissue at the root tip than older root tissue.

## 1.2 Strategies plants use to survive environmental stress

Everything in a plant's environment has the potential to become a source of stress. This means that under a particular set of environmental conditions a plant may have to adjust metabolism to optimize physiological performance in response to a stressor (Jansen and Potters 2017). Plant response to stress is mediated through a series of highly regulated strategies. Rhodes and Nadolska-Orczyk (2001) define *stress* in plants as a factor that constrains growth, productivity, reproductive success, and/or survival. In addition,

*strategy* can be defined as a genetically programmed response to stress that allows the plant to maintain or restore homeostasis (Hopkins and Hüner 2009). It is also important to note that this definition of *strategy* encompasses the entire mechanism of the response, which includes both signaling pathways and cross-talk between them, in addition to the regulation of gene expression (Gaspar et al. 2002; Hopkins and Hüner 2009). While this thesis focusses on suberin as a preformed barrier, the strategies plants use to combat stress are numerous and diverse, and it remains important to consider where preformed barriers fit into stress response as a whole. This includes considering the many elements that influence the strategy employed by a given plant to combat a specific stress.

Broadly, the response to stress is species dependent. For example, when comparing two Mediterranean gymnosperms in response to drought stress, *Pinus halepensis* avoids water loss through stomatal closure, where *Juniperus phoenicea* tolerates high water losses and is able to maintain a high rate of carbon assimilation under these conditions (Martínez-Ferri et al. 2000). This example also indicates that understanding response to stress is more than simply noting differences between species. The two species above differ in the approach employed to combat stress; avoidance vs. tolerance. Generally, mechanisms leading to stress *avoidance* prevent the plant from experiencing symptoms caused by the stress and are often mediated through a reduction in metabolic activity or state of dormancy (i.e. stomatal closure by *P. halepensis* prevents water loss; Gaspar et al. 2002; Hopkins and Hüner 2009). In contrast, mechanisms of stress *tolerance* allow the plant to maintain a threshold level of physiological function in the presence a given stressor (i.e. maintained carbon assimilation rate by *J. phoenicea* under drought conditions; Gaspar et al. 2002; Hopkins and Hüner 2009). In additional to being classified as either avoidance- or tolerance-based strategies, response to stress is also clustered by both the type of stress (abiotic or biotic) and where in the plant the stress acts or is perceived.

Abiotic stressors are environmental elements that are not derived from living organisms. They can be physical like temperature extremes, chemical like presence of a toxic compound, or mechanical like high winds (Rhodes and Nadolska-Orczyk 2001). In contrast, biotic stress occurs due to challenge by two main groups of organisms; pathogens and herbivores (Rhodes and Nadolska-Orczyk 2001). Regardless of the type of stress, in order for a response to occur the stress must first be perceived by the plant. This perception

occurs either in the aboveground structures of the plant (including stems, leaves, and reproductive organs like buds, flowers, and fruits) or in the roots (i.e. belowground structures including roots, tubers, stolons and associated structures like nodules). These broad-scale classifications aid in preliminary categorization of specific stress response strategies. For example, preformed barriers are avoidance-based strategies that can be found both above- and below-ground. Moreover, as preformed barriers protect against both water/nutrient loss and pathogen attack they play a role in both abiotic and biotic stress avoidance. However, while these broad-level strategy classifications help to distinguish between different stress responses, they fail to capture stress response in its entirety.

When considering response to stress, simply identifying the species of plant, approach of the response, type of stress, and site of perception, only begins to deconvolute the intricacies of the response. In addition to the type of stress, it is also important to consider whether the plant is experiencing a single stressor or a combination of stressors. For example, Arabidopsis challenged by two stressors in combination showed a differential response in gene expression where an average of 61% of differentially expressed genes detected by a combination of two stressors were not detected when the plants were challenged by a single stressor alone (Rasmussen et al. 2013). This added challenge is further complicated by intraspecies differences. For instance, cotton plants facing water deficit show an age-related response, where older leaves experience stomatal closure in response to water deficit sooner than younger leaves, which only experience stomatal closure under prolonged periods of stress (Jordan et al. 1975). Therefore, developmental stage of a specific tissue is important to consider in determining stress response. Stress response can also be tissue-specific. For instance, when undergoing hypoxia stress, Arabidopsis shoots require a functional aldehyde dehydrogenase (ADH) to avoid cytoplasmic acidosis, whereas root tissue remains hypoxia tolerant in *adh* plants (Ellis et al. 1999). It is important to remember that response to stress is genetically-based, and therefore responses can be genotype-specific. This is exemplified by Ahmed et al. (2013), where different strains of Tibetan barley (*Hordeum vulgar* subsp. *vulgare*) show contrasting capacities to produce osmolytes (e.g., proline) and protein stabilizers (e.g., glycine-betaine) and thus contrast in downstream capacity to combat combined drought and salt stress.

### *1.2.1 Response to abiotic stress*

The three abiotic stressors that have the highest impact on growth and productivity, and therefore the agricultural industry, are extreme temperature, drought, and salinity (Fraire-Velázquez et al. 2011; Gupta et al. 2014; Fang and Xiong 2015; Parihar et al. 2015). Generally, plant responses to extreme temperatures, drought, or high salinity are similar and lead to major impacts on plant physiology (osmotic and/or ionic stress, water deficit, and/or membrane disruption), which can ultimately lead to stress-induced injury (Tuteja 2009; Figure 1.1). Plants perceive abiotic stress through protein-based receptors that trigger signaling through second messengers (e.g. $Ca^{2+}$, ROS, and plant hormones) to ultimately elicit a genetically programmed response. This includes regulation of transcription factors (TFs) and activation of stress response genes. The activated mechanism then results in either an avoidance or tolerance-based strategy that leads to alterations in developmental, morphological, and/or physiological changes (Farooq et al. 2009; Fang and Xiong 2015; Heschel et al. 2017). These mechanisms are often shared between temperature extreme, drought, and high salinity responses.

In addition to the mechanisms to combat abiotic stress illustrated in Figure 1.1, preformed barriers (i.e. lignin, cutin, and suberin) are also known to play a role in response to abiotic stress. As reviewed in Le Gall et al. (2015), cell wall modification plays a role in plant response to abiotic stress, which includes an increase in cell wall lignification to increase stability and maintain physiological functions like turgor pressure. In response to drought, extreme temperature and salinity, there is evidence for increased lignification of both above- and below-ground tissues ( Le Gall et al. 2015). Cuticular waxes are also altered in response to abiotic stress. In Arabidopsis, salt treatment leads to an increase in the amount of wax per unit area from 32% to 80% and drought stress lead to a 65% increase in cutin monomer abundance (Kosma et al. 2009). As part of the plant response to abiotic stress, suberin deposition blocks apoplastic water transport, forcing a shift to transcellular water transport, which is more tightly regulated as it requires water transport proteins like aquaporins (Schreiber et al. 2005c; Chaumont and Tyerman 2014). Aquaporin expression

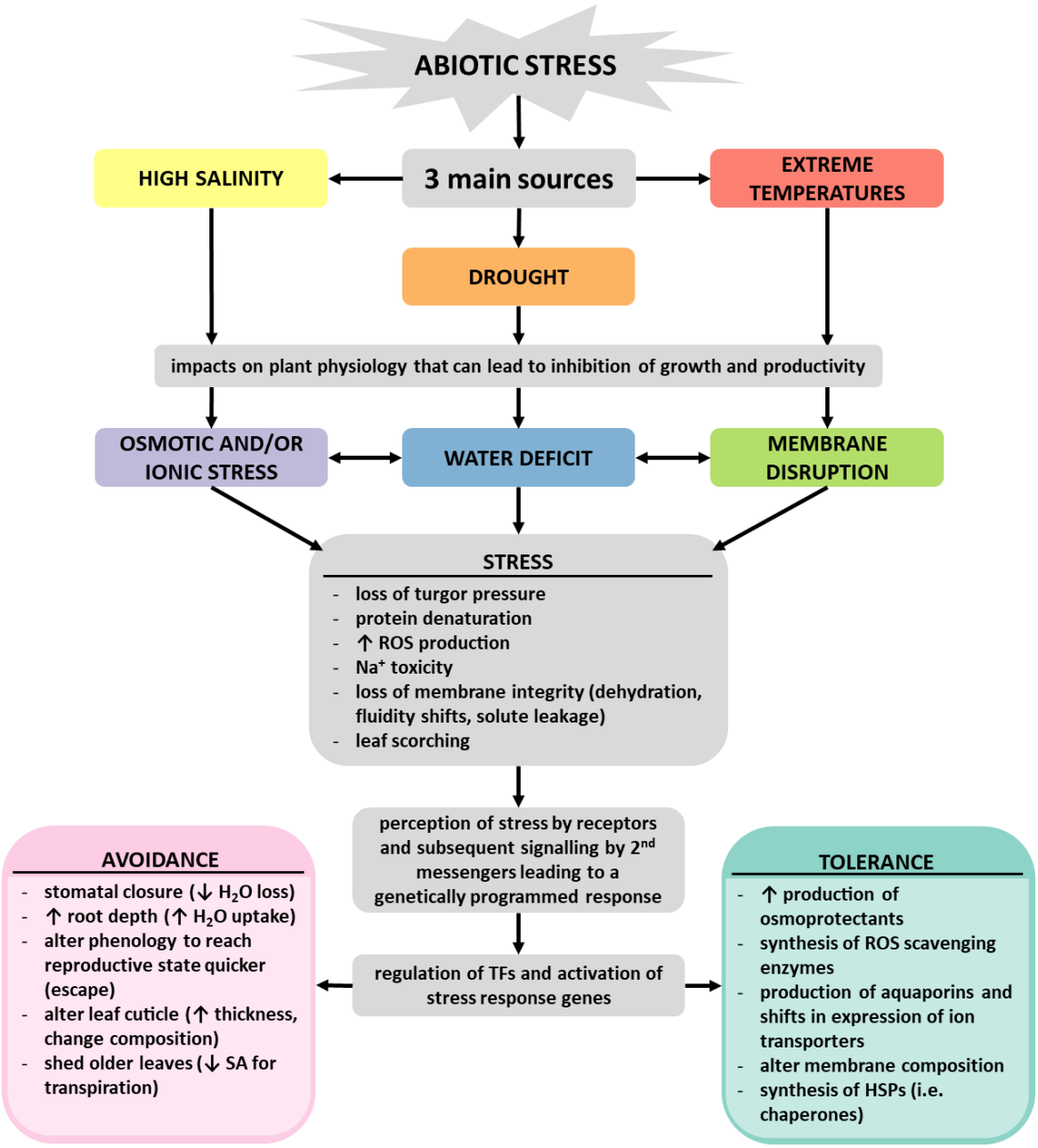


**Figure 1.1 Summary of major abiotic stressors and response mechanisms displayed by plants.** See text for details and Appendix A for references.

has been correlated with apoplastic barrier presence *in planta* (Chaumont and Tyerman 2014). Response to drought stress seems to be diverse. In soybean, drought stress results in morphological differences in suberin deposition where the number of suberized cell layers that make up the root epidermis increases from two layers to three layers (Makbul et al. 2011). In rice, plants grown under drought conditions shift the tissue-specific deposition of suberin from the sclerenchyma to the endodermis in order to increase the plant's ability to retain water (Henry et al. 2012). Drought can also lead to intraspecific differences in overall suberin abundance. For example, Holm oak (*Quercus ilex*) growing in forests that experience xeric conditions have increased total suberin aliphatics in their roots compared to those growing in forests that experience wetter conditions (Andreeta et al. 2013). In response to high salinity, shifts in suberin deposition generally seem to be developmental. In rice (*Oryza sativa*), cotton (*Gossypium hirsutum*), and common bean (*Phaseolus vulgaris*), deposition of suberin earlier in development and closer to the root tip occurs under conditions of salt stress, when compared to non-stress controls (Reinhardt and Rost 1995; Schreiber et al. 2005a; Krishnamurthy et al. 2009). This increase in suberin deposition in younger tissues was driven by increased abundance of ω-hydroxylated fatty acid monomers in all three species (Reinhardt and Rost 1995; Schreiber et al. 2005a; Krishnamurthy et al. 2009). In addition to a temporal shift in suberin development, cotton plants experiencing salt stress also developed an exodermis (including the deposition of suberin lamellae) not presence in control plants (Reinhardt and Rost 1995).

### *1.2.2 Response to biotic stress*

There are two main categories of biotic response in plants: (1) herbivory and (2) pathogen attack (Rhodes and Nadolska-Orczyk 2001). Herbivores are either vertebrate or invertebrate, and are generalists or specialists (Gong and Zhang 2014). Conversely, subdividing pathogens is more complex. A pathogen is any transmissible biotic agent that has the capacity to cause disease, and in plants can be foliar (infecting aboveground tissues) or soil-borne (infecting belowground tissues) (Agrios 2005a); this includes parasitic higher plants, nematodes, fungi, oomycetes, bacteria, and viruses. These biotic agents are also classified based on infection strategy, including biotrophic, hemibiotrophic, and necrotrophic infection (Doughari 2015). Biotrophs infect living plant tissues and cannot

survive in dead tissue, necrotrophs infect and kill plants and live off the dead tissue, and hemibiotrophs start off biotrophic and switch to a necrotrophic strategy as infection progresses (Doughari 2015). Plant responses to herbivory and pathogen attack can be similar but are still specific and relatively complex (Figure 1.2), therefore they will be discussed independently.

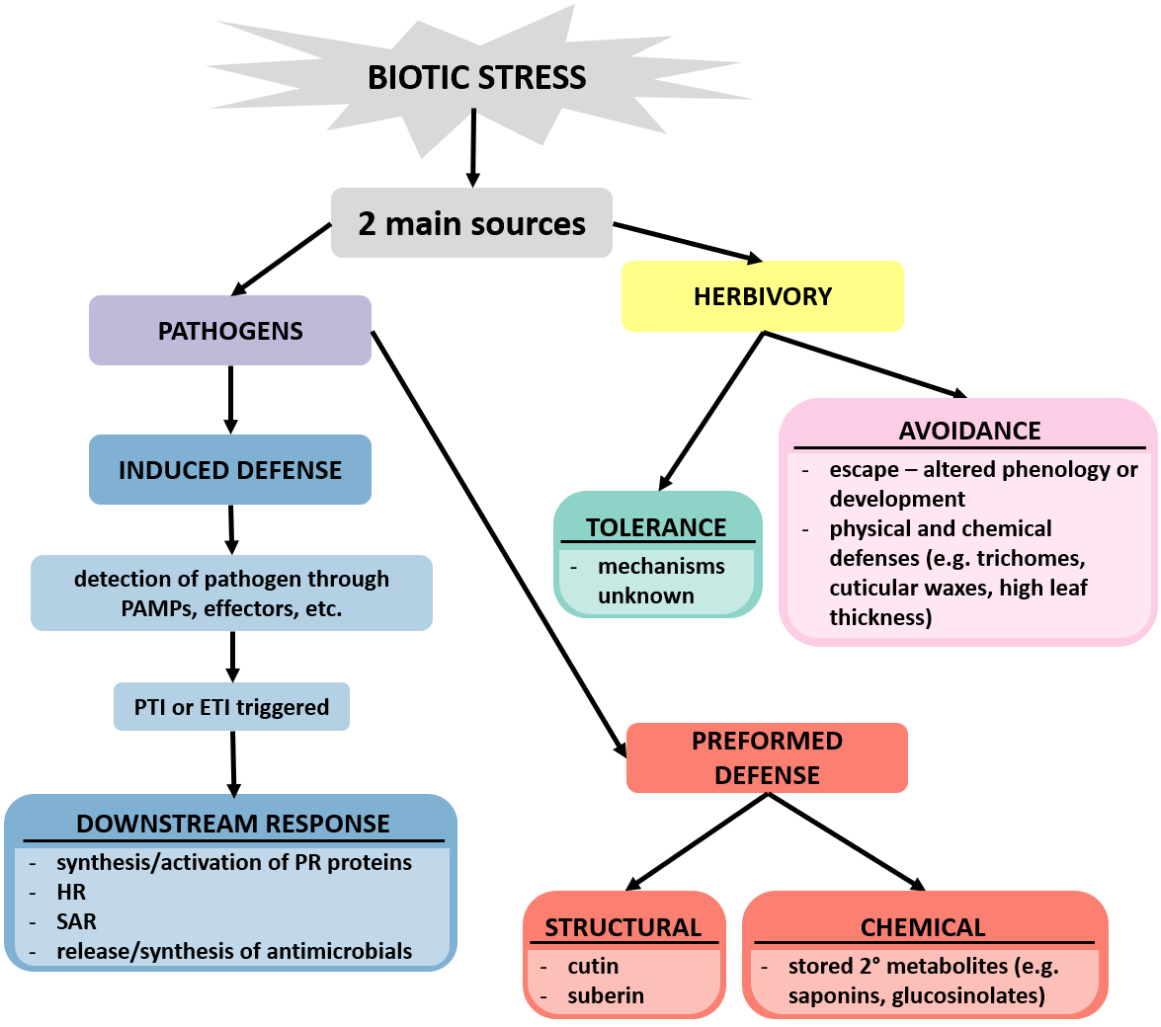


**Figure 1.2 Summary of major biotic stressors and response mechanisms displayed by plants.** See text for details.

#### 1.2.2.1 Response to herbivory

Plants have developed two main systems for defense against herbivores: (1) escape and (2) physical and chemical defenses (Gong and Zhang 2014). Escape relies on similar mechanisms such as in altered phenology and development. It is also hypothesized that trees able to disperse further away from the parent generate a protective effect from specialist herbivores, as the offspring will grow in a spatially distinct location (Gong and Zhang 2014). Defense against herbivory becomes more complex when considering physical and chemical defense strategies.

Physical defenses are structural and include trichomes, cuticular waxes, and high leaf mass to area ratios (i.e. high leaf thickness). Trichomes are very diverse in their function and structure, and their structural characteristics can be important in defense against herbivores. For example, in capture-events monitoring arthropod (*Liriomyza trifolii*) interaction with common bean, surface trichomes were shown to deter the organism's ability to walk, feed, and oviposit on the leaves (Xing et al. 2017). Cuticular waxes can also alter the topography to the leaf surface leading to similar impacts. High leaf thickness, usually relates to inedible leaves, where leaf thickness (and therefore toughness) is negatively correlated with herbivory (Gong and Zhang 2014). Chemical defenses are considered to be physiological and involved the production of secondary metabolites (including alkaloids, terpenoids, phenolics, plant hormones, protease inhibitors, and volatile compounds; Gong and Zhang 2014). These metabolites can have a direct impact on the herbivore, as is the case for most terpenes. Terpene biosynthesis is dependent on glandular trichomes. For example, tomato mutants deficient in glandular trichome production, and therefore decreased terpenoid content, showed decreased resistance to the herbivore, *Prodenia litura* (Gao et al. 2017). The impact of secondary metabolites can also be indirect by attracting natural enemies of herbivores. For example, when larval *Spodoptera exigua* feed on corn plants, the corn plants release a highly specific pattern of volatiles that attract parasitic wasps (*Cotesia marginiventris*) that parasitize the larvae (Turlings et al. 1990).

Most of the mechanisms described to combat herbivory can be classified as avoidance-based strategies, and little is known about tolerance to herbivory. Avoidance-based strategies have largely been identified based on observations of aboveground

herbivore interactions as research on below-ground herbivory is limited. However, there is some evidence of signaling between above- and below-ground tissues to impede further herbivory. For example, aboveground herbivory by corn leaf aphid (*Rhopalosiphum maidis*) results in systemic upregulation of the pathogenesis-related (PR) protein, resistance-cysteine protease, and leads to resistance against the belowground herbivore western corn rootworm (*Diabrotica virgifera virgifera*; Varsani et al. 2016).

#### 1.2.2.2 Defense against pathogens

Plant-pathogen interactions can be described in the context of a disease triangle with the pathogen, host, and environment all playing a role in the potential for infection (Agrios 2005b). Pathogens that are more virulent, active, and abundant are more likely to cause infection, and certain environments (e.g. wind, specific temperatures, high moisture) are more favourable to infection than others. Plants can directly impact the "host" side of the disease triangle based on the defense strategies they employ. That is, for infection to occur the interaction between plant and pathogen must be compatible. Incompatible reactions occur when a pathogen interacts with a non-host or a resistant-host. Regardless of the type of pathogen causing the stress, plants have evolved two main strategies for resisting infection: (1) preparing in advance (i.e. preformed defenses) and (2) waiting for attempted infection (i.e. induced defenses; Hammond-Kosack and Jones 1996). Induced defenses are activated at the time the stress is perceived, whereas preformed defenses are generated over the course of development and are present prior to interaction with a potential pathogen.

For induced defenses to be successful, recognition of the pathogen by the plant is crucial (Na and Gijzen 2016) to initiate signaling required for defense. As reviewed in De Coninck et al. (2015) and described briefly below, induced defenses are triggered in one of two ways: pattern-triggered immunity (PTI) or effector-triggered immunity (ETI). PTI involves recognition of pathogen-associated molecular patterns (PAMPs) by PAMP recognition receptors (PRRs; gene-for-gene interactions). PAMPs are highly conserved molecules within microbe classes that are important to microbe survival. Recognition of a PAMP by a PRR results in activation of defense or "immune" response in the plant. However, more successful pathogens have changed to suppress PTI through the evolution

of effectors that are usually injected into the cell and inhibit the signaling required for an immune response to occur. Plants subsequently evolved ETI to detect effectors used by pathogens through cytosolic R proteins that act as receptors. Both PTI and ETI have the potential to activate the synthesis of PR proteins as well as the hypersensitive response (HR) and systemic acquired resistance (SAR), but ETI is generally thought to be quicker in response and more robust in action than PTI (Thomma et al. 2011).

In response to post-recognition signaling transduction, there is a quick mobilization of defense responses including crosslinking of phenolics in the cell wall to strengthen and prevent movement to the next cell, as well as synthesis of PR proteins. The roles of individual PR proteins are highly diverse and include cell wall modification, synthesis of antimicrobials such as phytoalexins, and degradation of pathogen-specific cells wall components and proteins (Agrios 2005c; De Coninck et al. 2015). However, HR and SAR are characteristic responses that can be described more generally. HR refers to rapid cell death at the site of infection to prevent the spread of a pathogen (Durrant and Dong 2004; Agrios 2005c). It is initiated by release of toxic compounds including ROS and antimicrobials, as well as increased mobilization of ions and membrane disruption (Agrios 2005c). Together these physiological and metabolic shifts result in necrosis and eventual death of the infected cell(s) (Durrant and Dong 2004; Agrios 2005c). HR occurs on a much quicker time scale than SAR and therefore SAR generally occurs secondary to HR (Agrios 2005c). Whether as a result of HR or as a symptom of infection, cellular signaling in response to necrosis can trigger SAR (Durrant and Dong 2004). When triggered by HR, SAR signaling includes jasmonic acid (JA) derived from the oxidation of fatty acids during HR. JA signaling usually works in concert with salicylic acid (SA) signaling, the latter of which does not require HR to become active. These plant hormones trigger the synthesis of PR proteins, phytoalexins, and defensins (peptides that function in host defense) and upregulate defenses in a non-specific manner. Ultimately, this reduces the severity of the next attempted infection regardless of the pathogen. While all responses to pathogen attack have a genetic component, induced defenses tend to be more specialized in strategy than preformed defenses. As plants can experience interaction with a wide range of pathogens, they tend to rely on more general resistance strategies, or preformed defenses, for survival.

Preformed defenses fall into two main categories; chemical defenses and physical barriers. Chemical defenses include stored secondary metabolites, such as saponins or glucosinolates, that are toxic to the infecting pathogen (Osbourn 1996). These compounds are often sequestered in vacuoles or trichomes and are released at the time of infection by a pathogen. Perhaps more import are barrier molecules, as these are the first line of defense against pathogen attack (Doughari 2015). Barrier molecules in plants include the biopolymers cutin, and suberin. Cutin is the first line of defense against pathogens that infect aerial organs (Fich et al. 2016). For example, cuticle deficient tomato mutants display an increased infection occurrence by *Botrytis cinerea* than tomatoes with an intact cuticle (Isaacson et al. 2009). This increase in infection occurrence happens regardless of the genetic source of the mutant phenotype (Isaacson et al. 2009), which indicates defense against pathogens as a physiological function of the cuticle. The link between cuticle presence and pathogenicity is also indicated by observing mutant pathogens. When rice blast fungus (*Magnaporthe grisea*) is rendered cutinase deficient, the subsequent inability to degrade cutin delays infection of rice and barley by 2-3 days (Skamnioti and Gurr 2007).

Belowground, suberin provides roots with their first line of defense. Suberin is widely quoted as having a role in defense against pathogen attack (Beisson et al. 2012; Andersen et al. 2015; Doughari 2015; Vishwanath et al. 2015). In potato (*Solanum tuberosum*), wounded tubers that are given time to heal prior to inoculation gain complete resistance to *Erwina carotovora* subsp. *carotovora* (2-3 days pre-inoculation wound healing) and *Fusarium sanbucinum* (5-7 days pre-inoculation wound healing; Lulai and Corsini 1998). This indicates that a lack of a sufficient suberized layer leaves potato tubers vulnerable to attack. A similar response is seen with potato tuber pink eye infections, where histochemical analysis of both healthy and infected tubers revealed damage to the suberin barrier in infected tubers (Lulai et al. 2006). In soybean, the amount of preformed aliphatic suberin deposition is positively correlated with field tolerance against the pathogen *Phytophthora sojae*. That is, cultivars with a high abundance of aliphatic suberin also displayed low mortality in the field (Thomas et al. 2007). In addition, soybean cultivars with higher levels of suberin appeared to delay the infection process, as the pathogen requires an increased amount to time to overcome both the epidermal and

endodermal suberized layers in a cultivar with a high amount of suberin relative to cultivar with a low amount of suberin (Ranathunge et al. 2008).

Through analysis of plant interaction with the environment, at both the abiotic and biotic levels, the physiological role of suberin becomes clear. However, questions still remain regarding the specific compositional elements of suberin that are required for its different barrier properties. Therefore, to better understand the role of suberin in plant-environment interaction, a greater depth in the knowledge of the suberin structure, biosynthesis, and regulation of its deposition, are required.

## 1.3 Suberin

Suberin is the main physical barrier found in the belowground tissue (e.g. roots and tubers) of plants, as well as in the bark of woody plant species. It is a complex biopolymer, the deposition of which over the course of growth and development is a highly regulated and multifaceted process, leading to both tissue and age specific deposition patterns (Thomas et al. 2007; Beisson et al. 2012; Andersen et al. 2015). For example, in soybean roots, total suberin abundance varies along the root axis, where the younger tissue found near the root tip contains less total suberin than older tissue further along the root axis (Thomas et al. 2007). In addition, both suberin abundance and chemical composition vary when comparing suberin extracted from epidermal and endodermal layers of soybean roots (Thomas et al. 2007). Epidermal tissue is lower in total suberin, but shows a higher proportion of ω-hydroxylated fatty acids when compared to endodermal tissues (Thomas et al. 2007). The composition and abundance of suberin also depends on the species of the plant being studied (for examples of compositional differences in suberin deposition see the following: Arabidopsis, Höfer et al. 2008; potato Serra et al. 2009; rice and maize, Schreiber et al. 2005b).

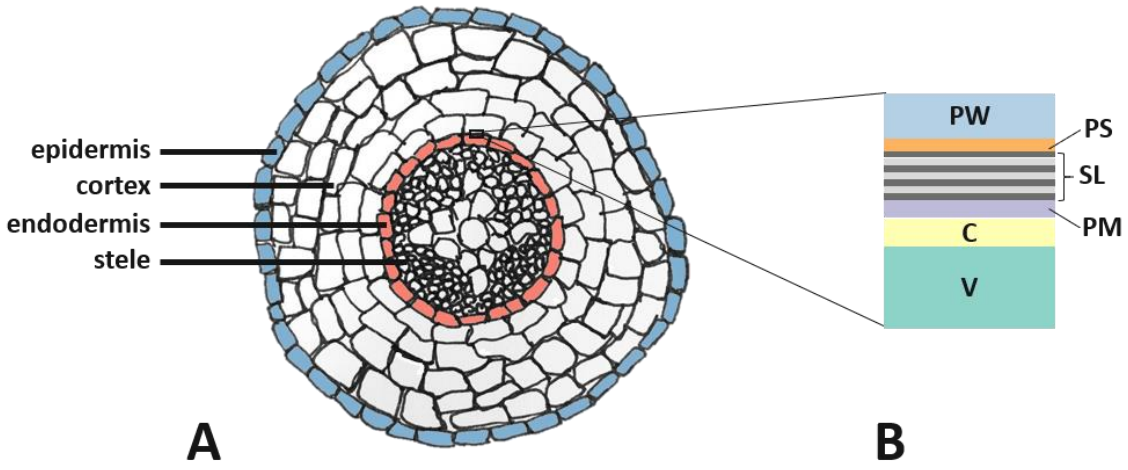


**Figure 1.3 Diagrammatic representation of localization of suberin deposition in root tissue.** (A) Cross section of root tissue. The epidermal layer is highlighted in blue and the endodermal layer is highlighted in red. (B) Schematic of subcellular localization of suberin deposition. V = vacuole, C = cytoplasm, PM = plasma membrane, PW = primary cell wall, PS = phenolic suberin, SL = suberin lamellae (aliphatic suberin).

### *1.3.1 Deposition, structure, and chemical composition*

Developmentally, suberin is characteristically deposited at one of two interfaces; tissue-tissue and tissue-environment (Vishwanath et al. 2015). In root tissue, this is seen in the deposition of suberin in the endodermal layer between the cortex and the stele, and epidermal layer (Bernards 2002; Thomas et al. 2007; Andersen et al. 2015; Vishwanath et al. 2015; Figure1.3A). In tubers, suberin is deposited in the periderm and helps form the skin of the tuber (Bernards 2002; Andersen et al. 2015). More specifically (based on potato tuber periderm), the phenolic domain is thought to be deposited in and covalently linked to the primary cell wall, while the aliphatic domain is thought to be deposited between the primary cell wall and the plasma membrane (Bernards 2002; Figure 1.3B). Specific dyes have been used to independently stain the phenolic (neutral red and toluidine blue 0) and aliphatic (fluorol yellow) suberin domains for visualization of tissue cross-sections using light microscopy (Lulai and Corsini 1998; Lulai et al. 2006; Thomas et al. 2007; Lulai and Morgan 2009). Transmission electron microscopy (TEM) has also been used to gain some insight on the macromolecular structure of suberin, revealing characteristic lamellar

patterns of aliphatic suberin which is arranged in highly consistent light and dark bands, or regions that are either electron light or dense (Kolattukudy 1980).

One structural characteristic of suberin is its hypothesized two-domain structure, wherein the phenolic domain and the aliphatic domain are deposited in the same cells of a given tissue, albeit in spatially distinct subcellular locations (Bernards 2002; Figure 1.4). It is also important to note that these two domains are covalently linked during the deposition of the polymer (Bernards 2002; Graça 2015; Vishwanath et al. 2015). While the exact structure of suberin remains unknown, partial depolymerization of suberin has resulted in some specific structural characteristics. Partial depolymerization analysis of total suberin has yielded small heteromers with both aliphatic and phenolic monomers linked to the same glycerol molecule giving evidence for the connection between the two

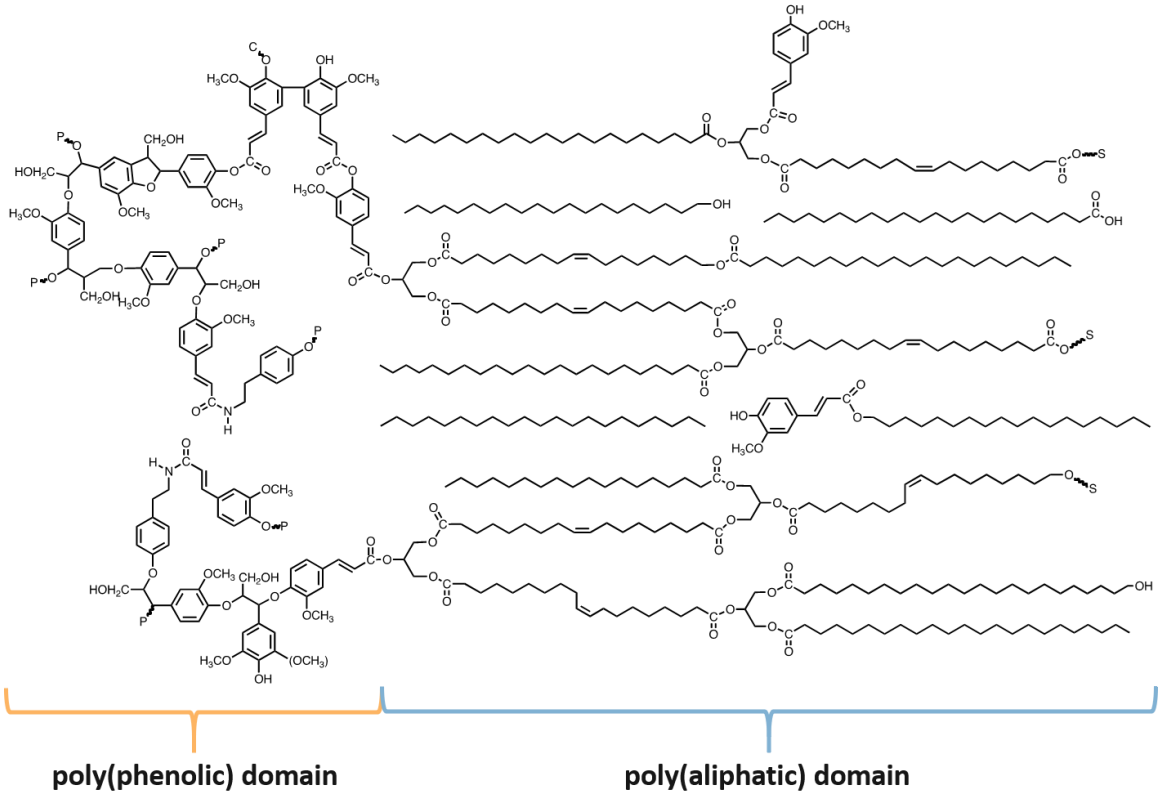


**Figure 1.4 Hypothesized two-domain structure of the biopolymer suberin.** This hypothesized structure is based on potato suberin and shows proposed linkages between monomers both within each domain (phenolic suberin on the left and aliphatic suberin on the right) and between the two domains. Adapted from Bernards, 2002.

domains (Graça et al. 2015). Therefore, regardless of whether the predicted subcellular location of the two domains is accurate, the cross-linking between the two domains remains definitive. And, it is this unique structural characteristic in combination with the localization of the biopolymer that likely results in its barrier properties.

As a barrier to water and solutes, suberin also functions in the regulation of water and solutes into the vascular tissue of plants. Apoplastic transport (diffusion within the hydrated cell wall and spaces between cells) of water and solutes is inhibited by the Casparian strip (primarily suberin) laid down on the radial walls between endodermal cells (Robbins et al. 2014; Andersen et al. 2015). Similarly, transcellular transport is inhibited by deposition of aliphatic, lamellar suberin around the outside of cells contributing to barrier layers (Robbins et al. 2014; Andersen et al. 2015). This indirectly allows for control of transport of physiologically important solutes including nutrients and toxins, as well as water. With both the apoplastic and transcellular pathways blocked through deposition of suberin, transport of water and solutes must occur through the symplastic pathway. By forcing symplastic transport, water and solutes must pass *through* the plasma membrane of non-suberized cells (i.e. passage cells) to access to the vascular tissue of the root (Robbins et al. 2014). Therefore, access to and from the vascular tissue can be regulated by the presence and/or activation of membrane bound transport proteins like aquaporins and ion channels, and the plant is therefore able to regulate access to the vascular tissue (Ranathunge and Schreiber 2011; Robbins et al. 2014; Vetterlein and Doussan 2016). This has important implications to the role of suberin in response to abiotic environmental stressors (see Chapter 1.2.1), as the plant is able to inhibit unwanted transport of water and solutes in or out of the cells and tissues. In terms of biotic stress, the barrier properties of suberin also play a role, especially in defense against pathogen attack (see Chapter 1.2.2). Pathogens often travel between the cells of plants, as in by apoplastic means, which is blocked by the deposition of suberin. Therefore, the pathogen must pass through any suberized layers in order to reach the vasculature of the plant. While suberin does not completely inhibit progression of the pathogen, it does slow the progression of the pathogen into the vascular tissue of the plant. This impediment of pathogen attack by suberin has the potential to ultimately result in a reduction in infection and therefore increased survival of the plant.

In addition to the localization and structure of suberin, compositional differences in suberin deposition have the potential to greatly impact the physiological properties of this barrier molecule. The importance of chemical composition to apoplastic barrier characteristics has been well described for cutin, where the relationship between cuticle thickness and permeability of the cuticle is non-linear (Norris 1974). A similar response is observed in potato tubers where wound periderm has water permeability 100-fold higher than its native periderm counterpart, despite containing suberin quantities equal to 60% of native periderm (Schreiber et al. 2005b). This indicates that chemical composition, and not just quantity, of suberin is important. To achieve a more detailed description of suberin composition, the polymer must be extracted from the plant tissue. Since isolating the suberin polymer intact is not readily possible, the details of structural and compositional elements have been examined through its partial to complete depolymerization. Based on this approach, and elaborated on more fully below, the phenolic domain is known to be compositionally similar to lignin albeit comprised primarily of hydroxycinnamic acids and their derivatives and to a lesser extent monolignols (which are the only monomers found in lignin; Bernards 2002; Graça 2015; Vishwanath et al. 2015). By contrast, the aliphatic domain is compositionally similar to cutin and is derived from primarily fatty acid-based monomers (Bernards 2002; Graça 2015; Vishwanath et al. 2015).

#### 1.3.1.1 The (poly)phenolic domain

The phenolic domain of suberin is comprised of both hydroxycinnamate and monolignol phenolic monomers derived from phenylalanine (Bernards 2002). The hydroxycinnamic *acids* have four main members ferulic, caffeic, and *p*-coumaric, and sinapic acid, while sinapyl, *p*-coumaryl, and coniferyl *alcohols* make up what are known as monolignols. These two types of monomers have the same core ring structure with different side chains and are synthesized sequentially with hydroxycinnamates acting as precursors for the synthesis of monolignols. These phenolic monomers are then linked together through the side chains to form polymeric phenolic suberin. In suberin, phenolic monomers are linked not only to each other, but are also linked to glycerol, and therefore the aliphatic domain of suberin, instead of additional phenolic components (Heldt and Heldt 2005).

#### 1.3.1.2 The (poly) aliphatic domain

Given the structural and compositional similarity between cutin and aliphatic suberin, knowledge of cutin biosynthesis (which is better characterized) can be used to inform hypotheses regarding suberin biosynthesis. Both of these barrier molecules are comprised of fatty-acid based monomers linked together through ester bonds with glycerol molecules as critical bridging linkages. While the macromolecular structure and monomer composition of these two polymers are similar, there are some clear distinctions between them (Andersen et al. 2015). At the polymeric level, these two barriers differ in their localization patterns. Cutin is deposited within and exterior to the primary cell wall on the surface of aboveground tissues including leaves, stems, and flowers. The deposition pattern of suberin differs from this in both tissue specificity and at the subcellular level. As indicated above, aliphatic suberin is deposited between the primary cell wall and the plasma membrane. Also, it is found in *both* above- (i.e. seed coat and woody tissues) and below-ground tissues (i.e. roots, tubers, and stolons). This same pattern of similar yet distinct characteristics also exists at the monomeric level.

The types of fatty acid monomers used to build these two polymers are generally the same and fall into one of three main sub-classes. First, unmodified, even chain length saturated fatty acids (with the exception of $C_{18:1}$). Second, fatty alcohols which consist of mainly primary alcohols. And third, oxidized fatty acids which include ω-hydroxylated fatty acids (ω-OHs), α,ω-dioic acids (DCAs), mid-chain hydroxylated fatty acids, and epoxy fatty acids. The ω-OHs and DCAs are particularly important, as these oxidized fatty acids play a key role in the polymerization of suberin as they are bi-functional and can be esterified at both ends. Where cutin and aliphatic suberin differ is in their specific monomer composition (Andersen et al. 2015). Cutin consists primarily of long chain length hydroxylated monomers whereas suberin tends to contain hydroxylated fatty acid monomers over a broad range of chain lengths, including very long chain. $C_{16}$ long chain hydroxylated monomers and two $C_{18}$ monomers (9,10,18-trihydroxysteric acid and 9,10-epoxy,18-hydroxysteric acid) are considered to be diagnostic of cutin, where $C_{16\text{-}26}$ even chain length DCAs are considered diagnostic of suberin (Nawrath 2002); however, these distinctions are not absolute as they can vary based on species and tissue type. Aliphatic suberin also contains, alkyl-ferulates (aliphatic monomers in linked to ferulic acid), which

along with some fatty acids, fatty-alcohols and alkanes function as suberin associated waxes (Bernards 2002).

The specific monomer composition of aliphatic suberin also varies across both species and tissue type. For example, aliphatic suberin in potato is characterized by a high abundance of DCAs, specifically of the chain length $C_{18:1}$, and contains monomers up to $C_{28}$ in chain length (Kolattukudy and Agrawal 1974). Compared to soybean, the compositional changes are minor; the $C_{18:1}$ chain length remains most common, however the overall proportion of DCAs is reduced, the ω-OH class of monomers are more predominant, and the maximum chain length of fatty acid derived monomers in soybean is $C_{24}$ (Thomas et al. 2007; See Chapter 3.2). A more drastic shift in composition is observed in corn, where the like soybean, ω-OHs are in highest abundance (Schreiber et al. 2005c). However, in corn the most common chain length *also* shifts to longer chain length monomers ($C_{22,24,26}$) (Schreiber et al. 2005c). Overall, while the three classes of aliphatic monomers are consistently found in aliphatic suberin, the specific composition is variable, indicating the importance of determining individual monomeric components in analysis, and not just total aliphatic suberin abundance.

While what we know about the structure and composition of suberin remains limited, it can still inform broad-scale features of this polymer. One example of this is that suberin is hydrophobic in nature and is insoluble *in vivo*. The insolubility of suberin is part of what makes it an effective barrier molecule, but it is still unclear whether the insolubility results from (1) the covalent anchoring to the cell wall, (2) the cross-linking of monomers in the aliphatic domain, or (3) the very high molecular weight; or some combination of the three (Beisson et al. 2012).

### *1.3.2 Biosynthesis and subcellular deposition*

Given the non-random and careful organization of suberin structure, biosynthesis is presumed to be a highly regulated process likely to proceed in a stepwise fashion. In an effort to simplify the processes and mechanisms involved in suberin biosynthesis can be categorized into; (1) phenolic metabolism, (2) aliphatic metabolism, (3) convergent metabolism, and (4) regulation. Phenolic metabolism refers to the biosynthesis of monomers for the phenolic domain, aliphatic metabolism refers to the biosynthesis of

monomers for assembly of the aliphatic domain, and convergent metabolism refers to the transport of monomers to the correct cellular location and linkage of monomers into the polymeric structure.

#### 1.3.2.1 Phenolic metabolism

Phenolic metabolism (Figure 1.5) starts with the shikimate pathway and the biosynthesis of phenylalanine, the initial substrate for the production of phenolic suberin monomers (reviewed in Lewis and Yamamoto 1990). During production of suberin monomers, phenylalanine is produced in the plastid, exported to the cytoplasm, and shuttled through the phenylpropanoid pathway. At the beginning of this pathway phenylalanine ammonia lyase (PAL) converts phenylalanine to *trans*-cinnamic acid; the first committed step in the metabolism of suberin phenolics. Next, *trans*-cinnamic acid is converted to *p*-coumaric acid by cinnamate 4-hydroxylase (C4H) which is further modified by hydroxylases and *O*-methyl-transferases to form caffeic, ferulic, and sinapic acids. These four acids make up the hydroxycinnamates and are converted to CoA-esters by 4-coumaroyl-CoA ligase (4CL). These CoA esters are either diverted towards convergent metabolism (see 1.3.2.3) or further modified by cinnamoyl-CoA-oxidoreductase (CCR) to form aldehyde intermediates. The aldehyde intermediates are used directly as substrate by coniferyl alcohol dehydrogenase (CAD) in the formation of the monoligonols, sinapyl, *p*-coumaryl, and coniferyl alcohol. The monolignols and hydroxycinnamates produced are the main monomers for phenolic suberin biosynthesis.

Once the monomers are generated, they also need to be transported to the primary cell wall and linked to both each other and the cell wall components. While the order of these steps remains to be elucidated, some hypotheses for the mechanisms behind each of these steps have been put forward. For example, the export of either monomers or preformed oligomers is hypothesized to occur through exocytosis where the phenolic precursors are first sequestered into a vesicle and subsequently exported by fusion with the plasma membrane (Lewis and Yamamoto 1990). Linkage of phenolic monomers is thought to be enzymatically driven by oxidases and/or peroxidases as is the case in lignin synthesis (Lagrimini 1991; Heldt and Heldt 2005), but characterization of these enzymes

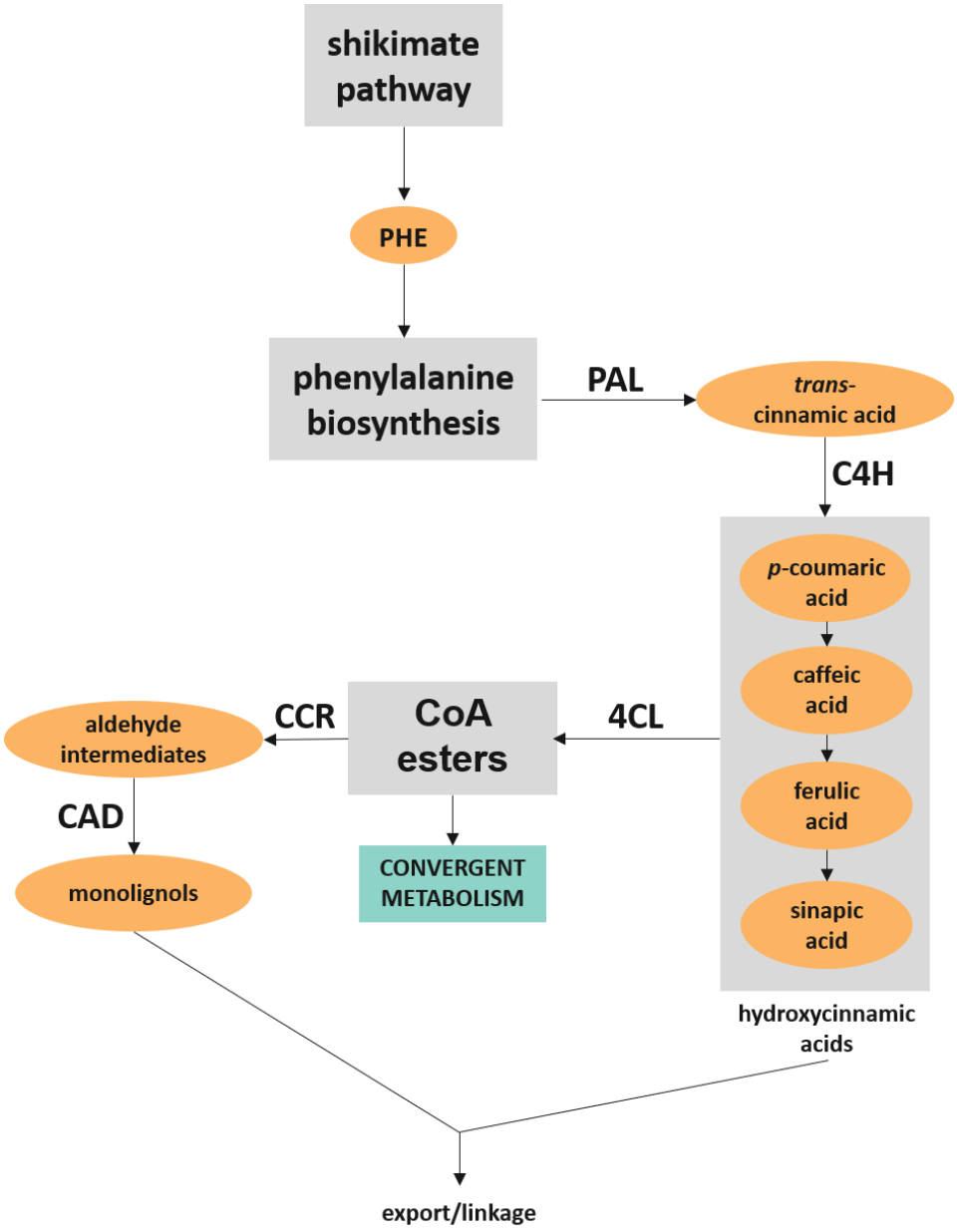


**Figure 1.5 Outline for metabolism of phenolic suberin monomers.** This illustration is general overview and does not include *all* details of each of the steps involved in phenolic suberin monomer biosynthesis. Molecules are highlighted in orange. Text over arrows refer to enzymes involved in metabolism between two molecules or molecule types.

and their role in suberin biosynthesis is limited. An acidic peroxidase has been spatially and temporary linked to suberization using a wound-healing potato model (Bernards et al. 2004). When isolated, this peroxidase shows substrate specificity for suberin associated phenolics *in vitro*, with a preference for hydroxycinnamic acids over monolignols (Bernards et al. 2004). This makes it a likely candidate for polymerization of the phenolic domain; however, analysis of this enzyme *in vivo* is lacking.

#### 1.3.2.2 Aliphatic metabolism

As with phenolic metabolism, the synthesis of the aliphatic domain also begins with the production of its monomeric components. Aliphatic metabolism (Figure 1.6) also begins in the plastid with the synthesis of long chain fatty acids (LCFAs) $C_{16}$ (palmitic acid), $C_{18}$ (stearic acid), and $C_{18:1}$ (oleic acid) fatty acids by the fatty acid synthase complex (Ohlrogge and Jaworski 1997). These initial aliphatic precursors are then transported to the endoplasmic reticulum (ER) and undergo one of two metabolic fates; (1) chain elongation into very-long chain fatty acids (VLCFAs) with even chain lengths greater than 18 carbons (with or without subsequent reduction to primary alcohols), or (2) oxidation of $C_{16}$ and $C_{18:1}$ fatty acids to generate either ω-hydroxylated fatty acids (ω-OHs) or dicarboxylic acids (DCAs) (Vishwanath et al. 2015). Chain elongated monomers may also be further modified to ω-OHs, and DCAs of longer chain length (Vishwanath et al. 2015). These steps are responsible for the production of the majority of the monomers that are incorporated in the aliphatic domain of suberin.

As *de novo* fatty acid biosynthesis is important to many different processes, the following details on aliphatic monomer synthesis begin with the modification of initial fatty acids including identification of enzymes responsible for each step. Chain elongation occurs by the addition of successive acetyl-units to pre-existing fatty acid chains and is controlled through ketoacyl-CoA synthases (KCSs). In potato, *StKCS6* knockdown lines resulted in a reduction of all monomers classes with chain lengths greater than $C_{28}$ (Serra et al. 2009a). Similar evidence for KCS mediated chain elongation exists in Arabidopsis where *kcs20 kcs2/daisy-1* double mutants also show a reduction in VLCFAs and their derivatives, specifically with chain lengths $C_{22}$ and $C_{24}$ (Lee et al. 2009). This confirms

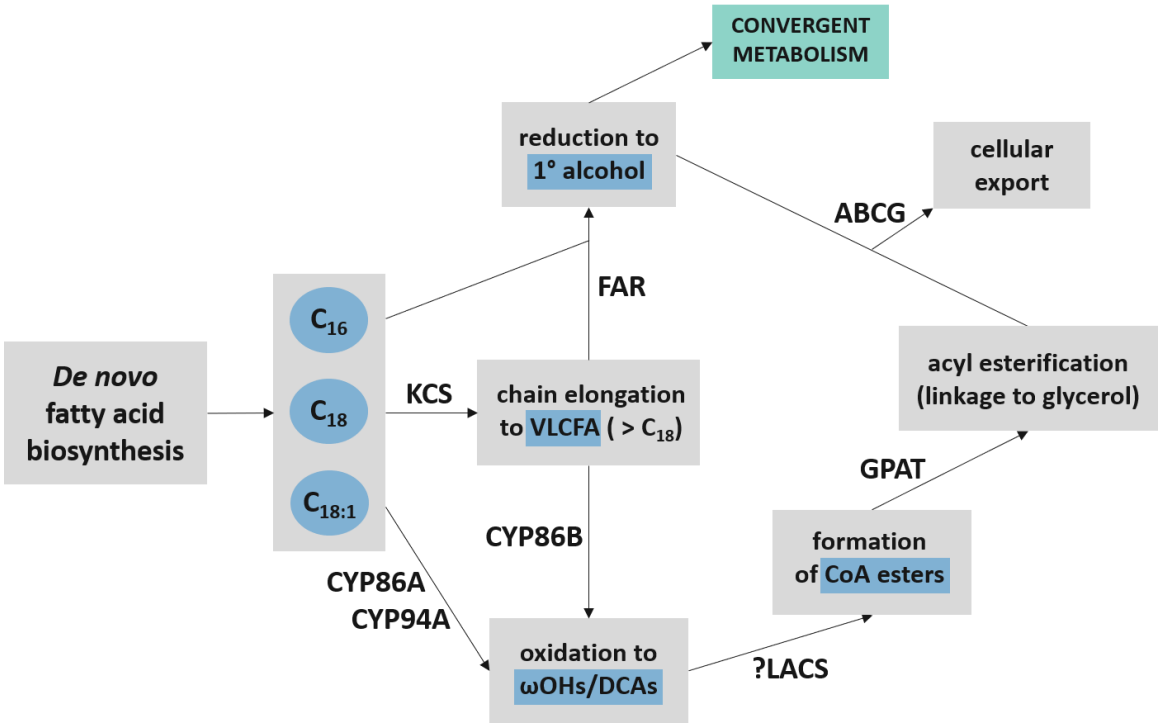


**Figure 1.6 Outline for metabolism of aliphatic suberin monomers.** This illustration is a general overview and does not include *all* details of each of the steps involved in aliphatic suberin monomer biosynthesis. Molecules are highlighted in blue. Text over arrows refer to enzymes involved in metabolism between two molecules or molecule types. The use of a question mark indicates that the enzyme involved in the reaction between two monomer types is hypothesized without any *in vivo* evidence for involvement in suberin biosynthesis.

involvement of KCSs in aliphatic suberin biosynthesis and indicates that more than one KCS would be required for suberin biosynthesis in each species, as different KCSs show different chain length specificities. In addition, KCS mutants show a reduced amount of all monomer classes of longer chain length, which indicates that elongation occurs before further modification of the fatty acid precursors (Lee et al. 2009; Serra et al. 2009a). Further modification of VLCFAs requires two other sets of enzymes.

Reduction of fatty acids into fatty alcohols is mediated by fatty-acid reductases (FARs). Like KCSs, FARs are also chain length specific. For example, in Arabidopsis three FARs have been characterized (AtFAR1, AtFAR4, AtFAR5; Domergue et al. 2010;

Vishwanath et al. 2015), each with a different chain length specificity. Through analysis of suberin monomers from FAR mutants, *far5* showed a reduction in $C_{18}$ primary alcohols, whereas *far4* and *far1* showed a reduction in $C_{20}$ and $C_{22}$ primary alcohols, respectively (Domergue et al. 2010). Putative suberin-related FARs have also been identified in other species, including StFAR3 in potato, giving strong evidence for the validity of this step in aliphatic suberin biosynthesis (Woolfson et al. 2018).

While similar evidence exists for the oxidation of fatty acids into ω-OHs and DCAs, the complete story is less clear. It is generally thought that fatty acids are oxidized sequentially; first to ω-OHs, then to DCAs (Kolattukudy 1981). These reactions are catalyzed by a family of cytochrome P450 enzymes known as the fatty acid ω-hydroxylases (FAωHs; Duan and Schuler 2005). For suberin biosynthesis, three subfamilies of FAωHs have been identified as having a role in the production of oxidized fatty acids (Figure 1.6). The first subfamily is the CYP86As that are thought to LCFAs and have been characterized for involvement suberin deposition in both Arabidopsis and potato (Höfer et al. 2008; Serra et al. 2009b). In Arabidopsis, the *AtCYP86A1* mutant *horst*, shows a reduction in ω-hydroxylated $C_{18:1}$ monomers as well as a reduction in overall suberin content (Höfer et al. 2008). This is mirrored in RNAi knockdown lines of *StCYP86A33*, where ω-hydroxylated $C_{18:1}$ and $C_{20}$ as well as total aliphatic suberin was reduced compared to wild-type (Serra et al. 2009b). Similarly, CYP86Bs are also responsible for ω-hydroxylation, but in this case of VLCFAs. This has been demonstrated in both Arabidopsis where AtCYP86B1 shows specificity for $C_{20\text{-}24}$ (Compagnon et al. 2009), and in rice where OsCYP86B3 prefers substrates of chain length $C_{24}$ or greater (Waßmann 2014). Conversely, CYP94A members are thought to catalyze the complete oxidation of fatty acids to DCAs. While some evidence for their involvement in suberin deposition has been indicated in *Nicotiana tobacum* (NtCYP94A5; Le Bouquin et al. 2001), the enzyme class is not widely accepted as being responsible for the production of DCAs that feed into suberin biosynthesis and it is unknown if the substrates for these enzymes are unmodified fatty acids or ω-OHs. While some further exploration is required to establish the details of DCA formation, generally the production of monomers for aliphatic suberin biosynthesis is well described. However, following this initial monomer synthesis, the steps involved in linkage and transport are less clear.

Linkage of aliphatic monomers to glycerol is achieved through enzymatic esterification which requires acyl-activation of one of the two substrates. Unmodified and oxidized fatty acids are first converted to CoA esters prior to linkage to glycerol and subsequent export from the cell. Fatty alcohols are generally thought to be exported without acyl activation, and in some cases, export is followed by direct linkage to phenolic monomers (as described in Chapter 1.3.2.3). In cutin synthesis, there is evidence for acyl activation of unmodified fatty acids by long-chain acyl-CoA synthetases (LACs; Schnurr et al. 2004), however, similar evidence is lacking in establishing a role for LACSs in suberin synthesis (Vishwanath et al. 2015). No evidence exists yet for LACS using oxidized fatty acids as a substrate. Despite the lack of evidence for specific enzymes responsible for acyl activation of these monomers, acyl activation itself is not an unfounded hypothesis. Structural analysis has indicated the presence of fatty acid derived monomers esterified to glycerol and GPATs (glycerol 3-phosphate acyltransferases) have been identified as, the enzymes responsible for this reaction. GPATs function by joining two monomers, one acyl donor and one acyl acceptor (Beisson et al. 2007; Li et al. 2007; Yang et al. 2012). In Arabidopsis, AtGPAT5 has shown a strong preference for $C_{16}$ and $C_{18:1}$ ω-oxidized fatty acids as substrate, which supports the hypothesis that fatty acid monomers are oxidized prior to esterification to glycerol (Beisson et al. 2007; Yang et al. 2012). There is also some evidence that depending on the GPAT, esterification to glycerol will occur in either an *sn-1* or *sn-2* position of glycerol-3-phosphate (Yang et al. 2012), which may give some hints as to the macromolecular organization of the aliphatic monomer components.

After ER associated esterification to glycerol, these newly formed suberin precursors, as well as the non-esterified fatty alcohols, must be transported out of the cell. Export seems to occur through the aid of specific transport proteins, specifically G-type ATP-binding cassette (ABCG) transporters. The strongest evidence for ABCG-based transport of aliphatic suberin monomers exists in potato and rice. In potato, *StABCG1* RNAi-knockdown lines show a reduction in monomers of chain length $C_{24}$ or greater, as well as the predominant aliphatic monomer components $C_{18:1}$ ω-OH and DCA (Landgraf et al. 2014). In rice, OsABCG5 shows a much higher substrate specificity, and only unmodified fatty acids and ω-OHs of chain length $C_{28}$ and $C_{30}$ are reduced in knockdown mutants (Shiono et al. 2014). As not all monomers are accounted for when knocking down

a single ABCG, it is likely that more than one ABCG in each species is required for export of *all* aliphatic suberin monomers.

#### 1.3.2.3 Convergent metabolism

Convergent metabolism refers to the bringing together of phenolic and aliphatic monomers and/oligomers to create suberin (Figure 1.7). In its most basic form, this can be thought of as the production of alkyl-hydroxycinnamates, where fatty alcohols from aliphatic metabolism are linked to hydroxycinnamate-CoA esters from phenolic metabolism (Bernards 2002). Enzymes for this reaction require specific hydroxycinnamates, but not primary alcohol chain lengths. For example, in Arabidopsis FACT is responsible for linking caffeoyl-CoA and primary alcohols (Kosma et al. 2012), where ASFT is responsible for linking feruloyl-CoA and primary alcohols (Molina et al. 2009). Identification of a similar enzyme for linkage to *p*-coumaryl-CoA has not yet been identified. The alkyl-hydroxycinnamate esters generated by these transferase reactions ultimately exist as suberin-associated waxes and are not covalently linked to the polymer (Bernards 2002). In potato *fht* (ASFT homolog) plants, the observed reduction in amount of alkyl-ferulates is not mirrored by an absence of lamellar suberin, indicating that a relatively high abundance of alkyl-ferulates is not required for the presence of a lamellar structure (Serra et al. 2010). Further knowledge of how the two domains are linked together is limited. Preliminary findings by Graça et al. (2015) suggest that linkage between the two domains likely occurs when ferulic acid and one or two long chain length ωOHs and/or DCAs are esterified to the same glycerol molecule. However, how this linkage occurs enzymatically is yet to be elucidated.

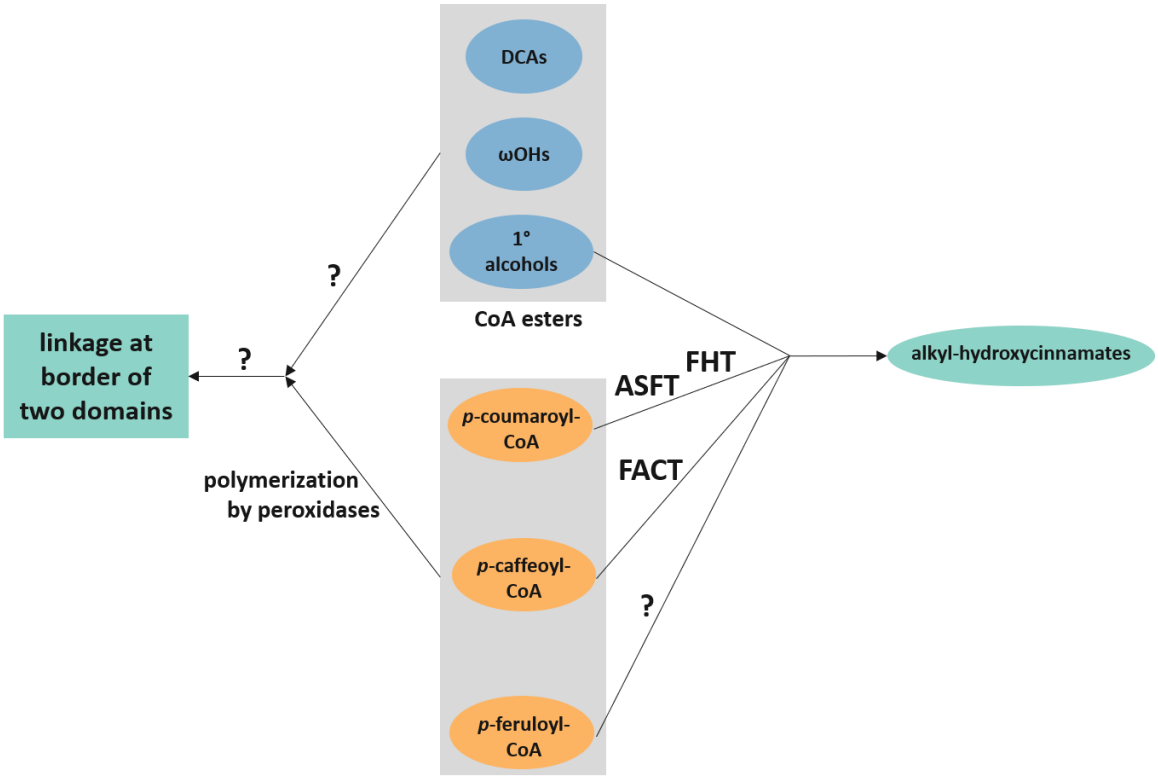


**Figure 1.7 Outline for convergence of phenolic and aliphatic suberin metabolism.** This illustration is a general overview and does not include *all* details of each of the steps involved in convergent metabolism. Molecules in orange are derived from phenolic metabolism whereas molecules highlighted in blue are derived from aliphatic metabolism. Molecules containing both aliphatics and phenolics are highlighted in green. Text over arrows refer to enzymes involved in the linkage of monomers. The use of a question mark indicates that the enzyme involved in the reaction for linkage of the monomers is unknown.

#### 1.3.2.4 Regulation

While acknowledging that any number of the regulation mechanisms may be involved in controlling the biosynthesis and deposition of suberin, recent research has focused on transcriptional regulation. It is also important to note phenolic metabolism and aliphatic metabolism are likely to be differentially regulated since the phenolic and aliphatic domains are spatially distinct. In a wounded potato tuber model of induced suberization, the phenolic domain of suberin was synthesized and deposited first (as it is responsible for anchoring the polymer to the cell wall). This was followed by a temporally

distinct deposition of the aliphatic domain, indicating the timing of activation of metabolism of these two domains is likely to be different (Yang and Bernards 2007)

When considering independent regulation of the two domains, there are three main TFs that have been identified (StWRKY1, AtMYB41, and StNAC103). First, for phenolic metabolism, phenylpropanoid metabolism is positively regulated by StWRKY1 in potato (Yogendra et al. 2015). However, whether or not StWRKY1-related increases in phenylpropanoid monomer production relates specifically to suberin biosynthesis is unclear (Yogendra et al. 2015). For aliphatic metabolism, developmental transcription factors remain elusive. Aliphatic suberin deposition is activated by AtMYB41 as indicated by increased expression of aliphatic suberin biosynthesis genes in over-expression lines (Kosma et al. 2014). Kosma et al. (2014) also demonstrated the activation of suberin biosynthesis by AtMYB41 at the phenotypical level, through the ectopic deposition of suberin lamellae and the presence of suberin specific aliphatic monomers *Nicotiana benthamiana* leaves expressing this TF. However, AtMYB41 is not a developmental TF and is only activated under abiotic stress conditions (Kosma et al. 2014). The first transcriptional repressor of suberin metabolism, StNAC103, has also been identified using wound healing potato tubers (Verdaguer et al. 2016). When *StNAC103* is silenced, an increase in both expression of aliphatic suberin biosynthesis genes and abundance of aliphatic suberin monomers is observed (Verdaguer et al. 2016). These three transcription factors regulate either phenolic or aliphatic suberin biosynthesis independently and support differential regulation of the two domains.

TFs that impact metabolism of both domains have also been identified. In opposition to the previously described TFs, AtMYB36 and MdMYB93 impact both phenolic and aliphatic suberin metabolism and argue against independent regulation of the two domains. In *myb36* plants, Casparian strip formation is disrupted as the absence of *AtMYB36* expression leads to insufficient localization of CASP1 (Kamiya et al. 2015). In addition to being Casparian strip deficient, *myb36* plants also display early deposition of suberin aliphatics, however how this is linked to decreased *AtMYB36* expression remains unclear (Kamiya et al. 2015). In apples, MdMYB93 is up-regulated in russeted apple skins compared to their non-russeted counter parts, and ectopic expression in *Nicotiana benthamiana* leaves results in the synthesis and deposition of both phenolic and aliphatic

suberin (Legay et al. 2016). In Arabidopsis, developmental regulation of seed coat suberin has also been linked to TFs, where AtMYB107 and AtMYB9 work together to synchronize the deposition of the phenolic and aliphatic domains (Costa et al. 2016; Hou et al. 2016). As the study of regulation of suberin biosynthesis progresses, it will be important to isolate TFs similar to the ones identified in tissues where suberin deposition is a crucial part of development (e.g. roots). In addition, considering whether transcriptional regulation is consistent across species, and whether it is sufficient in its regulation or requires additional levels of regulation (e.g. post-translational modification) would be prudent.

## 1.4 Thesis rationale

In the World Population Prospects 2017 report, the United Nations predicts a increase in global population to 8.6 billion by the year 2030, with continued increases over the course of the 21$^{st}$ century (United Nations, 2017). This increase in population puts a strain on current global food supply and security conditions, and necessitates improvements to food production and security policies. Crop improvement is likely to play a critical role in the ability to maintain food security in the future (Godfray et al. 2010; Godfray and Garnett 2014; Baldoni et al. 2015). Over the course of recent history, many improvements to agronomic practices have allowed for increased yield from crop plants, but they are likely to be insufficient to meet future demands. Especially in light of the changing climate, accessing the genetics realm of crop improvement will very likely be required to feed the future global population (Gross 2014).

One globally cultivated crop plant of interest is soybean. It is a highly versatile crop of great socioeconomic relevance including its role as the leading oil seed crop (accounting for 57% of plant sourced oil production; Hartman et al. 2015). Current soybean yields are estimated to be 60-80% of maximum with below maximum values being attributed to suboptimal growth conditions, including an 11% economic loss due to pathogen attack (Ramachandra et al. 2015). The pursuit of resistant cultivars has often focused on incorporating disease resistance genes into elite cultivars (i.e., gene-for-gene interactions; Hartman et al. 2015), which can be of limited affect (5-7 years) before the resistance is overcome through genetic variation in the pathogen (Schmitthenner et al.

1994; Abney et al. 1997). The ability of the pathogen to rapidly overcome resistant cultivars is highly problematic, so it has also been recommended to avoid reliance on a single method of resistance (Chang et al. 2017). In fact, no single soybean cultivar containing resistance genes is immune to all strains of *P. sojae* (Li et al. 2016). This has led to disease management strategies that focus on breeding high disease tolerance levels into new cultivars, rather than focusing solely on single resistance genes (Dorrance et al. 2003; Hartman et al. 2015). In this regard, it is important to understand the innate defense mechanisms employed by plants, and subsequently derive methodologies to enhance them in cultivars with otherwise desirable agronomic traits. Of the two categories of plant defense against pathogens that can be considered, induced defenses are typically mediated by gene-for-gene interactions (Hammond-Kosack and Jones 1996; Kamoun 2003) and have therefore been the focus of single resistance gene-based strategies for crop improvement. Instead, a greater emphasis could be placed on preformed defenses such as the protective barriers that are formed by biopolymers like cutin, lignin, and suberin (Beisson et al. 2012; Vishwanath et al. 2015) as these tend to be quantitative traits and offer multiple avenues for crop improvement that don't rely on a single gene-for-gene interaction. One of the major determinants of disease susceptibility is the ability to detect and respond to pathogens (Na and Gijzen 2016). By enhancing barrier molecules, like suberin, this creates a greater amount of time for the plant to detect and respond to the pathogen prior to infection, and likely has a role in reducing success of the pathogen.

## 1.5 Thesis objectives

For preformed barriers like suberin to be an effective target for crop improvement, there must exist a capacity for phenotypic improvement. Therefore, I have focused on gaining a better understanding of suberin biosynthesis using a genetics perspective. I first used a reverse genetics approach to investigate the function of two CYP86A genes using a hairy roots model system. This was followed by comparing monomer composition and gene expression during normal growth in development.

The first objective of my thesis was to identify putative fatty acid ω-hydroxylases (FAωHs) involved in the biosynthesis of soybean root aliphatic suberin deposition and

establish their function as FAωHs. I used a reverse genetics approach based on the RNAi-mediated knockdown of targeted genes coupled with detailed aliphatic suberin monomer analysis. To overcome difficulties typically experienced using traditional transformation techniques based on *Rhizobium radiobacter* (formerly *Agrobacterium tumifaciens*), we explored the use of a hairy root model system generated through *Rhizobium rhizogenes*-(formerly *Agrobacterium rhizogenes*) mediated transformation.

The second objective of my thesis was to determine the role of *GmCYP86A* genes in the developmental deposition of suberin in cultivars with different levels of suberin abundance. A comparative physiology approach was used to determine whether differences in suberin deposition and monomer composition correlated with changes in gene expression during normal growth and development. This was approached through analysis of aliphatic suberin deposition and gene expression across three cultivars (Williams, Conrad, and OX760-6) known to vary in suberin content.

# 2 MATERIALS AND METHODS

## 2.1 *In silico* identification of putative soybean ω-hydroxylases

To identify candidate fatty acid ω-hydroxylase (FAωH) genes in soybean, amino acid sequences of characterized CYP86A (AtCYP86A; Höfer et al. 2008), CYP86B (AtCYP86B1; Compagnon et al. 2009) and CYP94A (NtCYP94A5; Le Bouquin et al. 2001) enzymes, known to be involved in suberin biosynthesis, were used to conduct a BLASTp search of the soybean genome database on NCBI using default parameters. BLAST hits with an E-value of zero were selected for further phylogenetic analysis (Appendix B).

All amino acid sequences involved in the BLAST search, along with amino acid sequences from additional known CYP86As and CYP86Bs from Arabidopsis (Duan and Schuler 2005; Compagnon et al. 2009), rice (Krishnamurthy et al. 2009; Ranathunge et al. 2016), and potato (Serra et al. 2009), and CYP94As from tobacco (*Nicotiana tobacum*; Le Bouquin et al. 2001) and garden vetch (*Vicia sativa*; Benveniste et al. 2005), were aligned using MEGA 6 (Muscle algorithm; default parameters) with an additional cytochrome P450 enzyme (AtCYP83A) acting as the outgroup. The resulting alignment was used to construct a neighbour-joining tree using default parameters in MEGA 6 with 1000 bootstraps. The sequences that grouped with characterized CYP86As were selected for further analysis.

## 2.2 Tissue specific gene expression of putative CYP86As

RNA sequencing data (soybase.org; Grant et al. 2010) was compiled for 13 of the 14 putative soybean FAωHs identified *in silico* above (see Appendix C). RT-PCR was used to confirm RNA sequencing data from Soybase.org using gene specific primers designed by Koteles (2012), with roots and young leaves as tissue types (see Appendix D). Total RNA was extracted from approximately 1.0 g of each tissue type (roots and leaves) using a phenol:chloroform method modified from Sambrook et al. (1989). First a phenol:extraction buffer (50% phenol, 50% RNA extraction buffer (100 mM LiCl, 10 EDTA, 100 mM TRIS pH 8.0, 1% SDS) is prepared. This buffer is heated preheated to

80°C and then 0.1% β-mercaptoethanol and 0.2% pyrrolopyridine is added and the buffer is vortexed until homogenous. The buffer is then reheated at 80°C, and 5-8 mL was added to tissue previously ground in liquid nitrogen. The buffer tissue mixture is then vortexed for 5 mins (reheating for at least 30 seconds after 2.5 mins). A half volume of chloroform:isoamyl alcohol ($CHCl_3$:IAA) was then added, and the samples vortexed for 2 mins, and centrifuged for 20 mins at 12, 000 x g. The aqueous phase was collected and washed twice by vortexing for 2 mins with an equal volume of phenol:chloroform mix (50% phenol, 50% 24:1 $CHCl_3$:IAA), and a third time using a half volume 24:1 $CHCl_3$:IAA, using centrifugation (20 mins at 12, 000 x g) for phase separation and collecting the aqueous phase only each time. Next, an equal volume of 4 M LiCl was added to the final aqueous phase collection. Nucleic acids were precipitated overnight at -20°C, collected by centrifugation at 4°C for 30 mins at 12, 000 x g and then re-suspended in 400 µL DEPC-treated water. Nucleic acids were precipitated again using 1100 µL of 100% cold ethanol and 50 µL of 3.0 M $CH_3COONa$ before final resuspension in 30-60 µL of DEPC-treated $H_2O$. Prior to cDNA synthesis, equal amounts of all RNA samples were treated with DNase 1 (Fermentas) according to the manufacturer's protocol. DNase 1 treated samples were used directly for cDNA synthesis using SuperScript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The synthesized cDNA was then used as template for PCR with gene specific primers (PCR cycling conditions: 94°C for 5 mins; 28-32 cycles of 94°C for 50 secs, 55-65°C for 50 secs, 72°C for 1 min 30 secs; 72°C for 10 mins). Details for each gene can be found in Appendix D.

## 2.3 RNAi construct design

The design of the RNAi knockdown constructs for the root specific putative CYP86A genes in soybean (*CYP86A37* and *CYP86A38*) was done through several steps to help insure specificity to the target sequence and limit off-target effects. First, an alignment of the amino acid sequences CYP86A37 and CYP86A38 and well characterized CYP86As from *Arabidopsis thaliana* (AtCYP86A1) and *Solanum tuberosum* (StCYP86A33) was generated using DNAMAN. The alignment was subsequently analyzed to identify key domains including those shared between all cytochrome P450s and those shared between

members of the CYP86A subfamily (annotations were made according to: Werck-Reichhart et al. 2002; McGinnis et al. 2005; Schuler et al. 2006; Rupasinghe et al. 2007; Hlavica and Lehnerer 2010).

The annotations for common domains were mapped on to the corresponding nucleic acids in a DNA alignment of the exonic regions of the two soybean genes (DNAMAN; Appendix E). These regions of similarity were avoided in selecting sequence regions for RNAi construct building to avoid accidental knockdown of off-target genes. Next, the same DNA alignment was used to discern regions ideal for knocking down the genes independently versus in concert. The sequence alignment was scanned for regions of 21 nucleotides (nt) or greater that were near identical between the two sequences with a mismatch rate of $\leq 1$ mismatch in a single 21 nt region. This separated the sequences into two different categories. One with a high level of sequence identity and the potential to knockdown both genes, and one with lower sequence identity and single gene knockdown potential.

The annotated sequence alignment was used to select the 200-600 nucleotide fragments (Matthew 2009; Pandey et al. 2015) for generation of RNAi constructs. To have sequences *in vivo* that mimic pre-miRNA hairpin structure (an inverted repeat separated by an intron), the vector tool pKANNIBAL (Wesley et al. 2001; CSIRO, Austrailia; Appendix F) was incorporated into the cloning strategy. For cloning into pKANNIBAL, specially designed primers were used to amplify the regions of interest and add key restriction enzyme sites (see Appendix G) using full length gene clones as the template (PCR cycling conditions: 94°C for 5 mins; 35 cycles of 94°C for 45 secs, 55°C for 45 secs, 72°C for 1 min 15 secs; 72°C for 10 mins). The PCR-amplified fragments were cloned into pKANNIBAL and sent to the London Regional Genomics Centre (LRGC) to check for sequence identity and orientation using sequencing primers 5'-CACAATCCCACTATCCTTC-3' and 5'-CGTCTCGCATATCTCATTA-3'. The inverted repeats were then sub-cloned into the final destination vector, pHairyRed, which facilitated selection as transformed roots express a red fluorescent protein as a visual marker (Lin et al. 2011; Appendix H). pGEM (Appendix I) was used as an intermediate due to the limited availability of matching restriction enzyme sites between pKANNIBAL and pHairyRed. This generated three vectors *pHR37*, *pHR38* and *pHR37&38* that targeted

only *CYP86A37*, *CYP86A38*, and both *CYP86A37* and *CYP86A38*, respectively, which were used in the electroporation of *Rhizobium rhizogenes* (strain: LBA9402). For each electroporation a 50 µL aliquot of *R. rhizogenes* was thawed on ice and approximately 500 ng of plasmid was added once thawed. The *R. rhizogenes* and plasmid mixture was then transferred to a prechilled cuvette. The mixture was then pulsed twice for 2 seconds at the following settings: 25 µF capacitance, 2.50 kB, 400 Ω. The mixture was then incubated with 1 mL of LB for 1 hour at 28°C for recovery prior to plating on YMA with 50 µg/ml kanamycin for selection.

## 2.4 Generation of hairy root transformants

Hairy roots were generated using *Rhizobium rhizogenes* (strain: LBA9402) transformed with one of the following vectors: pHR37, pHR38, pHR37&38, or the empty pHairyRed vector (pHRempty) as a control. To obtain sterile cotyledons for inoculation with *R. rhizogenes*, soybean seeds (cultivar: OX760-6) were surface sterilized using 20% commercial bleach for 20 mins and subsequently rinsed 5 times with sterile Milli-Q® water. Surface sterilized seeds were plated individually on 1% sucrose agar and allowed to germinate at 25°C with a 12:12 hour (light:dark) photoperiod.

Once green (5-7 days post-plating), soybean cotyledons were inoculated by wounding the abaxial side of the cotyledon along the major vein with a scalpel previously dipped in a 36-48 hour *R. rhizogenes* culture. Once inoculated, the explants were placed on a solid Murashige-Skoog (M-S) medium (4.4 g $L^{-1}$ M-S salts (Sigma-Aldrich M0404), 3% sucrose, and 500 mg $L^{-1}$ cefotaxime (to prevent *R. rhizogenes* overgrowth); adjusted to pH 5.7-5.8 with NaOH) and placed at 25°C with a 12:12 hour (light:dark) photoperiod. Emergent roots (3-4 weeks post-inoculation), were screened for transformed roots using fluorescence microscopy (Zeiss StereoLumar V12 equipped with a 20 (Red) filter; excitation BP 546/12, emission BP 575-640) at the Biotron Imaging Centre (The University of Western Ontario). Roots that fluoresced red were sub-cultured separately on the same MS medium as above, as independent lines, and placed back in the incubator under several layers of cheese cloth to decrease the light intensity and prevent greening of the root tissue.

The independent lines were allowed to grow for an additional 6-8 weeks to obtain enough tissue for analysis.

## 2.5 Gene expression analysis of hairy root transformants

All root tissue for each transformant (not required for suberin analysis; approximately 0.4-1.0 g) was collected ensuring the removal of all media, flash frozen in liquid nitrogen and stored at -80°C prior to extraction. RNA extraction was performed as described in Chapter 2.2, and all RNA samples were analyzed spectrophotometrically using a NanoDrop ND-2000 (Thermo Scientific) to determine concentration and sent to the LRGC for bioanalyzer analysis. RNA samples with a RIN score ≥ 7.5 were used for DNase 1 treatment and cDNA synthesis as described above. Synthesized cDNA was diluted 4-fold prior to use as a template in qRT-PCR analysis.

For quantification of *CYP86A37* and *CYP86A38* expression in transformants relative to empty vector controls, qRT-PCR was performed using iTaq™ Universal SYBR® Green Supermix (Biorad) and a CFX96™ (Bio-Rad) real-time detection system. Gene specific primers were designed, and standard curves were conducted using a mixture of cDNA templates serial diluted 5-fold each time from the original concentration for a minimum of 3 points to determine primer efficiencies (see Appendix J). The amplification of *CYP86A37* and *CYP86A38* was normalized using the ΔCq method with the control genes *cons6* and *cons7* (Libault et al. 2008). Each of the three primer pairs was run in triplicate for all transformant and control samples, and the ΔΔCq method in the Gene Study package (Bio-Rad CFX Manager™ Software Version 3.1) was used to determine relative expression of the two genes of interest between individual transformants and the control group.

For analysis correlating gene expression to suberin content, an alternative expression value calculation of the same data was generated. In this case, all samples including the controls were treated as individual samples rather than as groups. To achieve these individual expression values the ΔΔCq method in the Gene Study package was still used, however, the "relative to none" feature was selected instead of generating values relative to a specific sample.

## 2.6 Characterization of soybean hairy root suberin

Aliphatic suberin monomer profiles of hairy roots and soil-grown roots were generated using GC-MS. Aliphatic suberin was depolymerized, extracted, and derivatized using a "no-extraction" method according to Bjelica et al. (2016). To attempt to control for developmental differences in aliphatic suberin content, only the first 10 cm (measured from the root tip) from the main root of either the hairy root culture or from 10-14 day old soil grown seedlings were used for analysis. The lateral roots were trimmed, and the remaining 10 cm piece was air dried between two paper towels (until paper towels appeared dry; less than 15 minutes) prior to being ground in liquid nitrogen to a fine powder. The ground tissue was transferred to a glass vial and its mass recorded (4.67 ± 1.45 mg). Next, 0.5 mL of 3 M methanolic HCl was added and the vials incubated at 80°C for 2 hours. The vials were allowed to cool prior to the addition of 0.5 mL NaCl-saturated $H_2O$ to stop the reaction. Internal standard, (10 µL of 1 mg $mL^{-1}$ triacontane in chloroform) was added, and the total aliphatics extracted with hexane (1.0 mL) three times. The hexane extracts were pooled and evaporated under nitrogen gas prior to derivatization with 50 µL each of pyridine and 99% BSTFA + 1% TMS for 40 min at 70°C. Methyl ester/TMS ether derivatives (1 µL) were injected (splitless) into a Varian CP-3800 Gas Chromatograph equipped with a flame ionization detector (GC-FID) and a Varian MS220 ion trap Mass Spectrometer (GC-MS). Capillary GC on CP-Sil 5 CB low bleed column 0.25 mm ID, 30 m, 0.25 µm (CP7860 Agilent) was conducted under following conditions: 4 min at 70 ºC, 40 ºC/min to 200 ºC, 2 min at 200 ºC, 3 ºC/min to 320 ºC, held 10.75 min at 320 ºC, for a total run time of 60 min. Monomers were identified based on their electron-impact MS spectra (70 eV, m/z 40 – 550). Compound abundance was quantified from the GC-FID chromatograms and normalized to the internal standard.

## 2.7 Plant material for analysis of different cultivars

Soybean seeds of three different cultivars (Conrad, Williams, OX760-6; 2013 seed source) were obtained from Dr. Mark Gijzen at Agriculture and Agri-food Canada (London, ON). Waxed paper cups (8.5 cm diameter x 15 cm deep; Merchants Paper Company, Windsor, ON, Canada) with a single drainage hole cut into the bottom, were

used as pots. Thirty-six seeds of each cultivar were planted (one seed per pot) in hydrated vermiculite (Therm-O-Rock, Inc.) and placed in a growth chamber with the following conditions: 25°C, 16h:8h (light:dark) photoperiod, 60% relative humidity. Plants were watered from below using deionized water and monitored for emergence. Plants that did not emerge at the same time as the majority were discarded.

Root tissue was collected 10-days post-planting. The plants were gently up-rooted, rinsed in deionized water to remove any remaining vermiculite. Lateral roots were trimmed from the main root, and the main root was segmented into three sections; tip (0-4 cm), middle (4-8 cm), and top (8-12 cm). For RNA extraction, tissue from 15 plants was pooled together for each section ($n$ = 1). Tissue to be used for RNA extraction was immediately flash frozen with liquid nitrogen and then stored at -80°C until analysis. For suberin analysis, three biological replicates were collected. Tissue from three plants was pooled together for each replicate and the fresh weight of the pooled tissue was determined prior to flash freezing and storing at -20°C until analysis ($n$ = 3). The complete experiment was repeated 3 times.

## 2.8 Gene expression of tissue from different cultivars

RNA was extracted from a total of 18 samples; tip, middle, and top from each cultivar (OX760-6, Conrad, and Williams) with three biological replicates each time the experiment was repeated. Pooled, collected tissue from each sample was ground using liquid nitrogen, and RNA was extracted using the phenol:chloroform based method described in Chapter 2.2 Extracted RNA was assessed by spectroscopy (NanoDrop ND-2000; Thermo Scientific) to determine quantity and aliquots were stored at -80°C. One aliquot of each sample was sent to the LRGC for bioanalyzer analysis and all samples had excellent RIN scores (RIN $\geq$ 8.7). Stored samples were then treated with DNaseI (Fermentas), and DNaseI-treated samples were used directly in cDNA synthesis using SuperScriptII reverse transcriptase (Invitrogen; as described in Chapter 2.2). Prior to RT-qPCR analysis, successful cDNA synthesis was confirmed through conventional RT-PCR for *ACT-II* (Hu et al. 2009; Table S3.1; PCR cycling conditions: 94°C for 2 min; 35 cycles of 94°C for 30 s, 61°C for 45 s, 72°C for 45 s; 72°C for 5 min).

All qPCR analysis was performed using iTaq™ Universal SYBR® Green Supermix (Biorad) and a CFX96™ (Bio-Rad) real-time detection system. Prior to use in qPCR analysis, cDNA was diluted 4-fold. First, standard curves for all genes were generated to determine primer efficiencies (Appendix K). Equal aliquots of all 18 samples were combined and further diluted 5-fold in series to generate a total of five concentrations. Each concentration was analyzed in triplicate for a total of six genes; two target genes *CYP86A37* and *CYP86A38*, and four candidate reference genes, *ACT-II*, *cons4*, *cons6*, and *cons7* (Table S3.1; Libault et al. 2008). Based on primer efficiencies, *cons4* and *cons7* were selected as references genes moving forward.

Next, expression of the two genes of interest *CYP86A37* and *CYP86A38*, and the two reference genes *cons4* and *cons7* were measured in triplicate. Melt curves were generated after completion of the amplification cycles to ensure amplification of a single product. Relative expression of *CYP86A37* and *CYP86A38* between samples was determined using the ΔΔCq method and the Gene Study package in Bio-Rad CFX Manager™ (Software Version 3.1) using the relative to none feature (as no sample acts as a clear control). When considering gene expression profiles between individual samples, biological replication was not included in the "plate" file in order to obtain individual values for each biological replicate and be able to determine standard deviation between replicates. However, when examining the relationship between gene expression and suberin content, biological replicate information was included in the "plate" file to obtain a single value for each sample type.

## 2.9 Characterization of suberin across cultivars

Tissue collected for suberin analysis was ground using liquid nitrogen and transferred to pre-weighed 2 mL screw-cap tubes and weighed to determine the mass of the tissue (88.1 ± 16.5 mg). First, to remove any soluble metabolites, a two-phased extraction was preformed based on the method described by Shepherd et al. (2007). Tissue was kept frozen until the addition of 300 µL of methanol per sample. Samples were then incubated at 30°C for 30 minutes, vortexing at 2 min intervals. Next, 75 µL of 0.02 mg $mL^{-1}$ ribitol (dissolved in water), 600 µL of chloroform, and 10 µL of 1 mg $mL^{-1}$ triacontane

(dissolved in chloroform) were added sequentially to each sample, and the samples vortexed. A second incubation at 30°C for 30 minutes (vortexing at 2 min intervals) followed. To encourage phase separation, 150 µL of water was added to each sample and the samples and shaken by hand to mix. Samples were centrifuged at 16,000 x g for 10 min. Following centrifugation, 200 µL aliquots each of the top, polar phase, and bottom, organic phase, were transferred to independent glass vials. Phase aliquots were then dried under nitrogen and stored at -20°C to be saved for analysis at a later date.

To recover tissue for suberin analysis, remaining solvent from both phases was carefully removed and samples were rinsed in 500 µL of acetone by vortexing and then centrifugation at 16,000 x g for 5 min. Solvent was discarded, and the samples washed a second time in 500 µL of acetone, this time incubating in acetone for 10 mins prior to centrifugation at 16,000 x g for 10 min. Solvent was discarded, and the tubes were left open to air dry in the fume hood. Dried tissue was then weighed into glass vials and suberin monomers were extracted, derivatized, and analyzed by GC-MS as described in Chapter 2.6. Potato reference material was included with each batch of soybean samples for quality control.

## 2.10 Statistical analysis

All statistical analyses were conducted in R (3.5.3) with a significance level of $\alpha = 0.05$. For aliphatic suberin analysis, samples were analyzed individually; either individual hairy root lines (both empty vector controls and RNAi knockdowns) or single samples from the three cultivars. Monomers characteristic of soybean aliphatic suberin were identified based on mass spectra and quantified by converting raw area values obtained by FID to mass values using external calibration curves. These values were then normalized to the mass of tissue extracted. Normalized values for individual monomers were summed to obtain values of total aliphatics, as well as the monomer subclasses (unmodified fatty acids, fatty alcohols, oxidized fatty acids which includes ω-hydroxylated fatty acids and dicarboxylic acids).

For specific analysis of ω-hydroxylated fatty acids from hairy roots samples, values for all ω-hydroxylated fatty acids were summed to obtain a total, and normalized monomer

values were used directly when considering single chain lengths. The relationship between suberin components and gene expression (Figure 3.7) was evaluated using the "cor.test" function with the individual gene expression scores extracted from the Gene Study package analysis as the x-variable and the normalized molar amount of suberin as the y-variable. Individual ω-hydroxylated monomers were also analyzed using the "t.test" function to look for differences between control roots and RNAi knockdown lines generated with the pHR37&38 construct.

For samples from the different cultivars, monomer class sums and individual monomer values were averaged across biological replicates within an experimental replicate ($n = 1$). The average values from each experiment were used in comparing overall suberin profiles between cultivars and root segments as well as in determining the relationship between gene expression and suberin deposition ($n = 3$). For comparison of oxidized fatty acids between cultivars, the total amount of individual monomers was summed along the root axis prior to analysis. Overall suberin and gene expression profiles (values for individual biological replicates were used here) were evaluated using a two-way RM-ANOVA with cultivar and root segment as factors using the "aov_ez" function from the "afex" package. Post-hoc analysis to determine where differences occur for the RM-ANOVA was executed using the "emmeans" function from the "emmeans" package. To determine whether there were differences in oxidized fatty acids between cultivars, a one-way MANOVA with cultivar as a factor and the six individual monomers as variates was conducted using the "manova" function. All relationships between aliphatic suberin deposition and gene expression were evaluated using the "cor.test" function using normalized average values for each sample type (extracted directly from the Gene Study package analysis with biological replicates included in the plate file) as the x-variable and the normalized molar amount of suberin as the y-variable.

# 3 RESULTS

## 3.1 Fatty acid ω-hydroxylases in soybean

In focusing on oxidized fatty acids and the fatty acid ω-hydroxylases (FAωHs) that catalyze their formation, the phylogenetic relationship between amino acid sequences of soybean candidate genes and representative CYP86As, CYP86Bs, CYP94As from other land plants was investigated. This revealed several candidate homologues for the same subfamilies in soybean (Figure 3.1).

Of the six soybean candidates that cluster within the CYP86A clade, two (*GmCYP86A37* and *GmCYP86A38*) were most closely aligned with genes known to encode enzymes involved in suberin biosynthesis: *AtCYP86A1* (Höfer et al. 2008) and *StCYP86A33* (Serra et al. 2009b). The remaining four sequences clustered together with CYP86As involved primarily in cutin biosynthesis (see for example, Pinot and Beisson 2011). The three *GmCYP86B* genes clustered together, forming a clade with an uncharacterized *CYP86B1*-like gene from *S. tuberosum*. Importantly, *AtCYP86B1*, from which the *S. tuberosum* gene derives its name, has been characterized as suberin-associated (Compagnon et al. 2009). Lastly, the *GmCYP94A* genes aligned closely with suberin-associated genes from *V. sativa*, either alone (i.e., *GmCYP94A20* with *VsCYP94A2* and *VsCYP94A3*) or in a distinct clade most closely associated with *VsCYP94A1*. RNA-Seq data compiled from Soybase.org revealed a tissue specific expression pattern where *GmCYP86A37, GmCYP86A38, GmCYP86B9, GmCYP86B10, GmCYP94A17* and *GmCYP94A19* all showed highest expression in roots and/or nodules, while the other soybean CYP candidates were either expressed only at low levels in below ground tissues or were predominantly expressed in aerial tissues (see Appendix C).

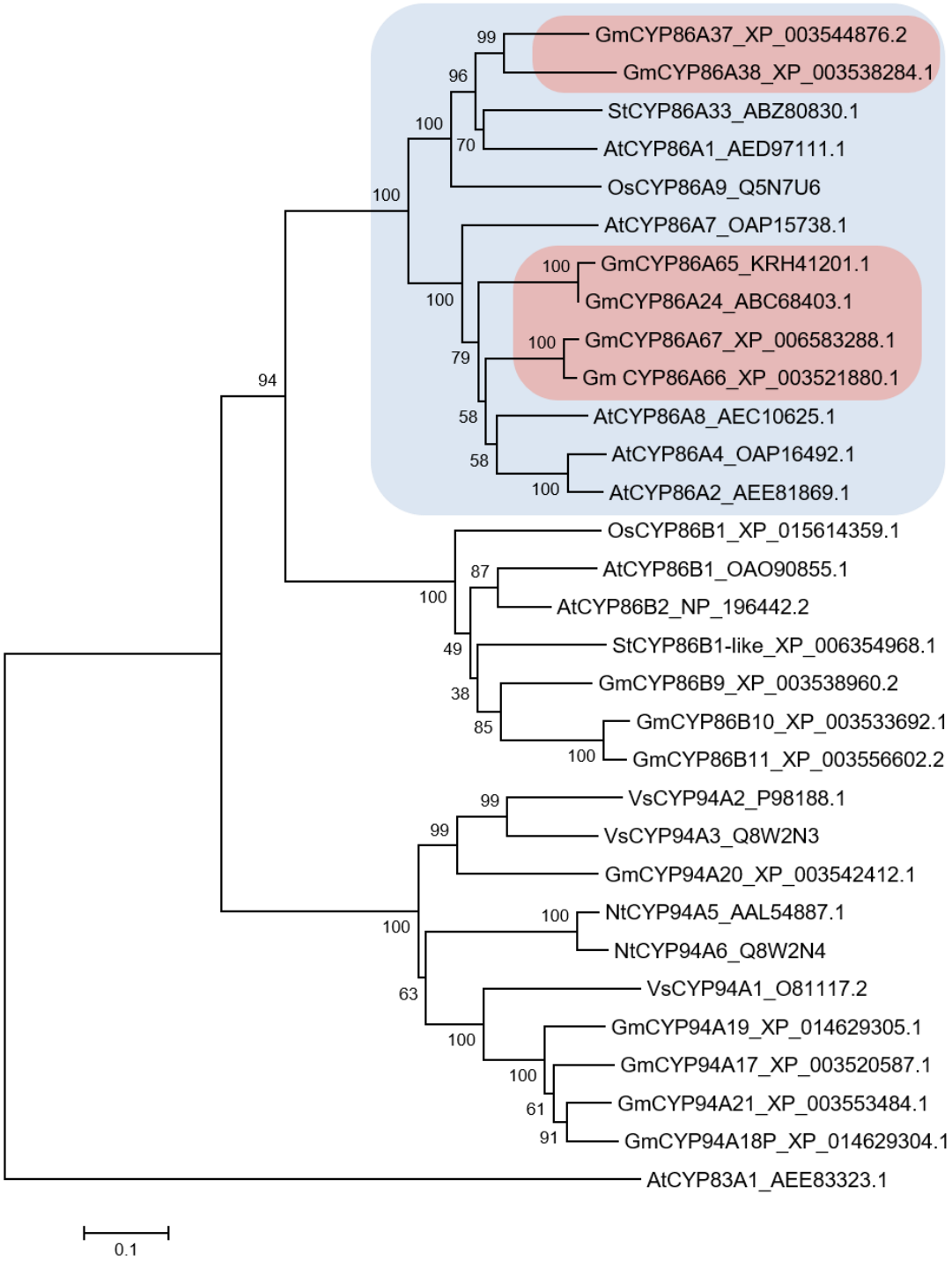


**Figure 3.1 Phylogenetic tree of CYP86A, CYP86B, and CYP94A families.** Sequence annotations are in the format of enzyme name followed by NCBI accession number (At = *Arabidopsis thaliana*, Gm = *Glycine max*, Nt = *Nicotiana tobaccum*, Os = *Oryza sativa*, Vs = *Vicia sativa*). The CYP86A subfamily is highlighted in light blue, with the soybean candidate genes highlighted in light red. All sequences were aligned using MEGA 6 (Muscle algorithm), and the resulting alignment was used to construct a neighbour-joining tree with 1000 bootstraps.

Since soybean aliphatic suberin is dominated by long chain ω-OHs, and AtCYP86A1 has been shown to prefer 18-hydroxy-oleic acid as substrate (Benveniste et al. 1998), we focused our attention on putative soybean members of the CYP86A subfamily. As this family contains six putative members in soybean, we first confirmed the root-specific expression of *GmCYP86A37* and *GmCYP86A38* by RT-PCR to narrow down candidate genes that have the potential to impact suberin deposition in soybean roots (Figure 3.2A,B). Thus, based on their phylogenetic relationship to suberin specific enzymes, the predicted substrate preference based on AtCYP86A1, and their tissue specific expression patterns, *GmCYP86A37* and *GmCYP86A38* were selected as targets for RNAi-knockdown.

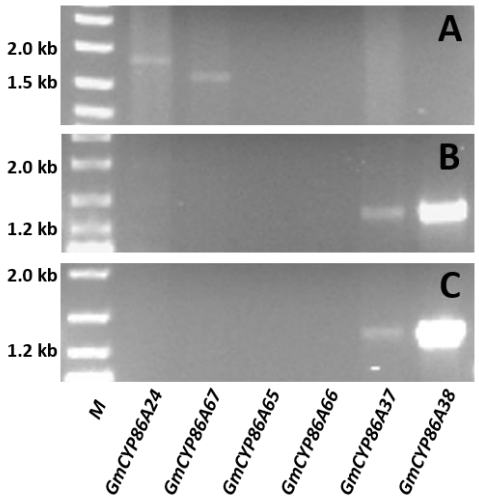


**Figure 3.2 Visualization of RT-PCR displaying the expression pattern of putative soybean CYP86As.** mRNA isolated from (A) leaves, (B) soil-grown roots and (C) hairy roots, was reverse-transcribed into cDNA and used a template for RT-PCR. Gene specific primers (see Appendix B) were used to measure the transcript levels of six *GmCYP86A* genes. Lanes are labelled below the gel images. M = marker with specific bands labelled with sizes for reference.

## 3.2 Characterization of soybean hairy roots

Since soybean is not very amenable to whole plant transformation (Kereszt et al. 2007; Cao et al. 2009; Li et al. 2010; Mangena et al. 2017), and suberin deposition occurs in roots, we used a hairy root system to investigate soybean FAωHs. To ensure that this was an appropriate system to study root suberin deposition in soybean, we first characterized the chemical composition of hairy root suberin and compared them with soil-grown roots. Aliphatic suberin analysis of soil-grown soybean roots shows that soybean aliphatic suberin is primarily composed of long chain ω-hydroxy fatty acids (ω-OHs) and dicarboxylic acids (DCAs; Figure 3.3). These same compounds were present as the major

aliphatic suberin monomers in soybean hairy root tissue, albeit with minor differences in the ratio of DCA:ω-OH. In hairy roots, DCAs are proportionally higher than in soil-grown roots, especially 16:0 DCA (Figure 3.3). Regardless, 18-hydroxy-oleic acid remained the predominant compound regardless of root type. In addition, the gene expression pattern of the six putative GmCYP86As, matched that of soil grown roots with only *GmCYP86A37* and *GmCYP86A38* expressed in hairy roots (Figure 3.2C).

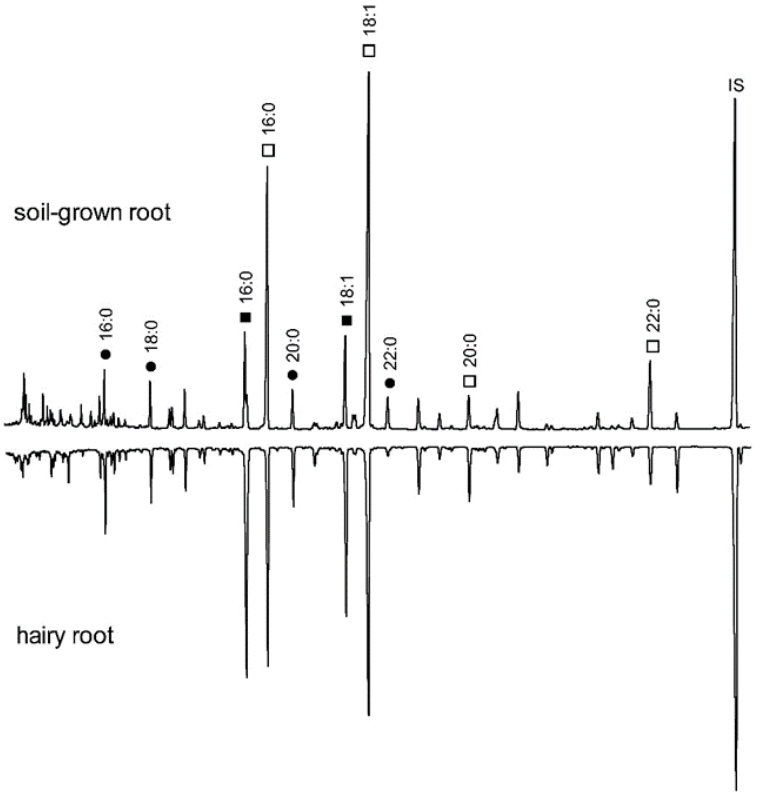


**Figure 3.3 Representative gas chromatograms of aliphatic suberin monomers from soil-grown and hairy roots.** Aliphatic suberin monomers were derived from whole roots using a no-extraction protocol based on depolymerization using methanolic-HCl and derivatization using BSTFA-TMS, followed by GC-MS analysis. Relevant peaks are labelled according to monomer class (● fatty acids, □ ω-hydroxy fatty acids, ■ dicarboxylic acids) and chain length. IS = internal standard.

## 3.3 Phenotypic characterization of RNAi knockdown lines

To establish the physiological role of the putative FAωH genes in suberin deposition in soybean, we used a reverse genetics approach involving RNAi knockdown of two root-specific soybean CYP86A genes (*CYP86A37* and *CYP86A38*). We attempted to generate RNAi-knockdown lines targeting *GmCYP86A37*, *GmCYP86A38* both individually and in combination. Despite numerous attempts, we did not recover any knockdown lines using the construct targeting *GmCYP86A38* alone (data not shown) and only one line using the *GmCYP86A37* construct was recovered. By contrast, lines generated using the construct targeting both *GmCYP86A37* and *GmCYP86A38* were readily recovered. RT-qPCR analysis of both transformed lines and empty vector controls indicated successful knockdown of at least one of the two genes in the lines selected for further analysis (Figure 3.4).

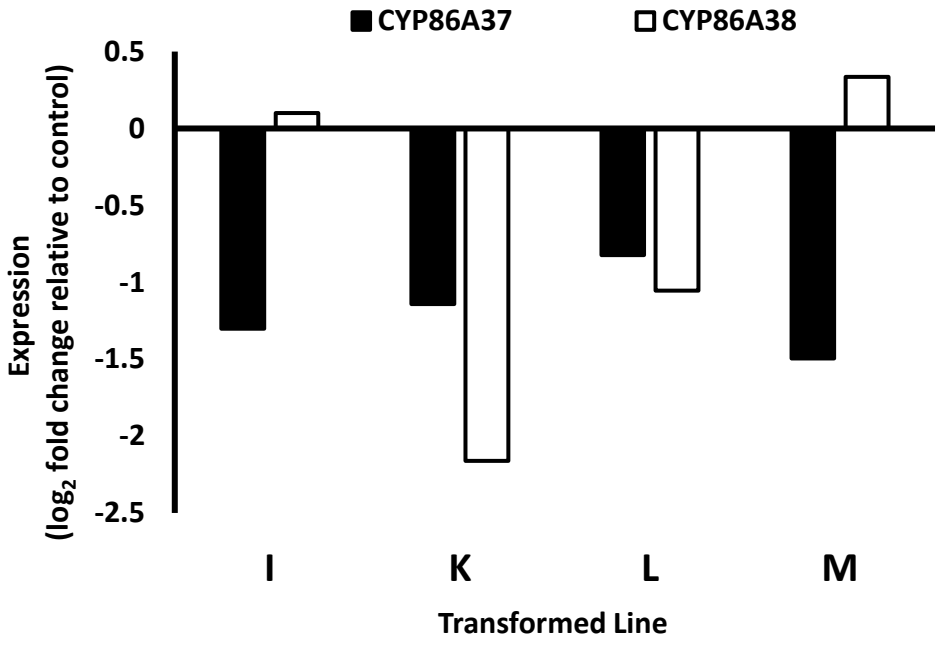


**Figure 3.4 Expression of *GmCYP86A37* and *GmCYP86A38* in independent RNAi knockdown lines.** The relative expression of *GmCYP86A37* (filled bars) and *GmCYP86A38* (open bars) in RNAi knockdown lines was determined by comparison to empty vector control lines using the $\Delta\Delta C_q$ method in the Gene Study package (BioRad CFX Manager™ Sotware Version 3.1) with *cons6* and *cons7* as reference genes. Values represent expression of individual transformants relative to the control group after $\log_2$ transformation to allow for better visualization. Line I was transformed using *pHR37*only, where Lines K-M were transformed using *pHR37&38*.

Following gene expression analysis, aliphatic suberin was measured for all hairy root lines. To determine whether decreased expression of *CYP86A37* and *CYP86A38* had an impact on aliphatic suberin abundance, the relationship between gene expression and total aliphatic suberin abundance was tested. There was no significant relationship between total aliphatic suberin and expression levels of either *GmCYP86A37* or *GmCYP86A38* (Table 3.1, Figure 3.5). To determine the impact of reduced *CYP86A37* and *CYP86A38* expression on suberin composition, the aliphatic monomers were grouped based on compound class, including unmodified fatty acids, fatty alcohols, or oxidized fatty acids. Similar to total aliphatic suberin content, no significant relationship between gene expression and aliphatic suberin content was observed for any of the three monomer classes (Table 3.2, Figure 3.6).

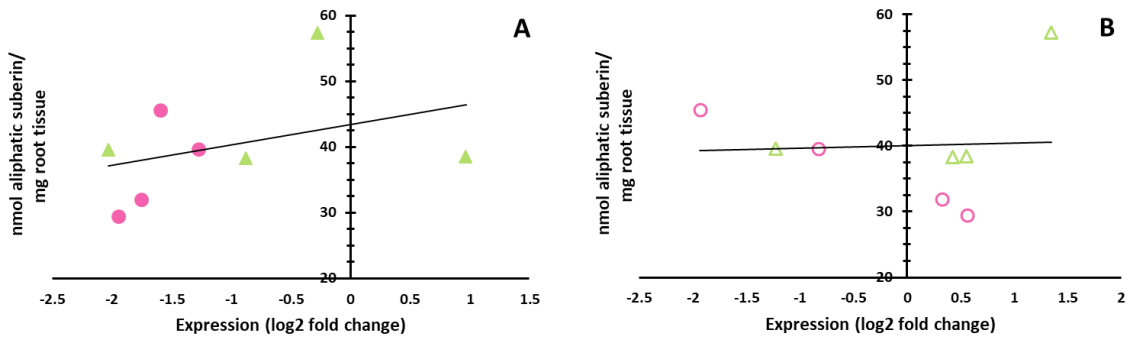


**Figure 3.5 Relationship between the expression of *GmCYP86A* genes and total aliphatic suberin.** The expression of *GmCYP86A37* (A) and *GmCYP86A38* (B) is plotted relative to total aliphatic suberin measured in the same tissues. Triangles represent controls, circles represent RNAi-knockdown lines. Each data point represents an independent transformed hairy root line, including empty vector controls. Expression data are presented as $\log_2$ fold change, relative to the two reference genes (*cons6* and *cons7*). Trend lines are for visualization only. See Table 3.1 for statistics summary.

**Table 3.1 Summary of correlation analysis of CYP86A gene expression and total aliphatic suberin content in hairy roots.**

| X- variable | Y-variable | Correlation Coefficient | 95% Confidence Interval | P-value |
|---|---|---|---|---|
| *CY986A37* expression | Total aliphatic suberin | 0.3625 | -0.4595, 0.8500442 | 0.3775 |
| *CY986A38* expression | Total aliphatic suberin | 0.0313 | -0.6885, 0.7201 | 0.9413 |

**Table 3.2 Summary of correlation analysis of CYP86A gene expression and monomer subclass content in hairy roots.**

| X- variable | Y-variable | Correlation Coefficient | 95% Confidence Interval | P-value |
|---|---|---|---|---|
| *CY986A37* expression | Unmodified fatty acids | 0.2630 | -0.5421, 0.8164 | 0.5291 |
| | Fatty alcohols | -0.1972 | -0.7918, 0.5894 | 0.6397 |
| | Oxidized fatty acids | 0.5129 | -0.3004, 0.8943 | 0.1937 |
| *CY986A38* expression | Unmodified fatty acids | -0.1009 | -0.7521, 0.6500 | 0.8121 |
| | Fatty alcohols | -0.3542 | -0.8474, 0.4670 | 0.3893 |
| | Oxidized fatty acids | 0.2749 | -0.5330, 0.8206 | 0.5099 |

**Table 3.3 Summary of correlation analysis of CYP86A gene expression and ω-hydroxylated monomer content in hairy roots.**

| X- variable | Y-variable | Correlation Coefficient | 95% Confidence Interval | P-value |
|---|---|---|---|---|
| *CY986A37* expression | $C_{16:0}$-ωOH | 0.0655 | -0.6701, 0.7362 | 0.8776 |
| | $C_{18:1}$-ωOH | 0.6597 | -0.0840, 0.9314 | 0.0751 |
| | $C_{20:0}$-ωOH | -0.0790 | -0.7423, 0.6626 | 0.8526 |
| | $C_{22:0}$-ωOH | -0.2532 | -0.8129, 0.5495 | 0.5451 |
| *CY986A38* expression | $C_{16:0}$-ωOH | 0.0620 | -0.6720, 0.7346 | 0.8840 |
| | $C_{18:1}$-ωOH | 0.7427 | 0.0798, 0.9501 | **0.0348** |
| | $C_{20:0}$-ωOH | -0.3987 | -0.8614, 0.4255 | 0.3279 |
| | $C_{22:0}$-ωOH | -0.1885 | -0.7884, 0.5953 | 0.6549 |

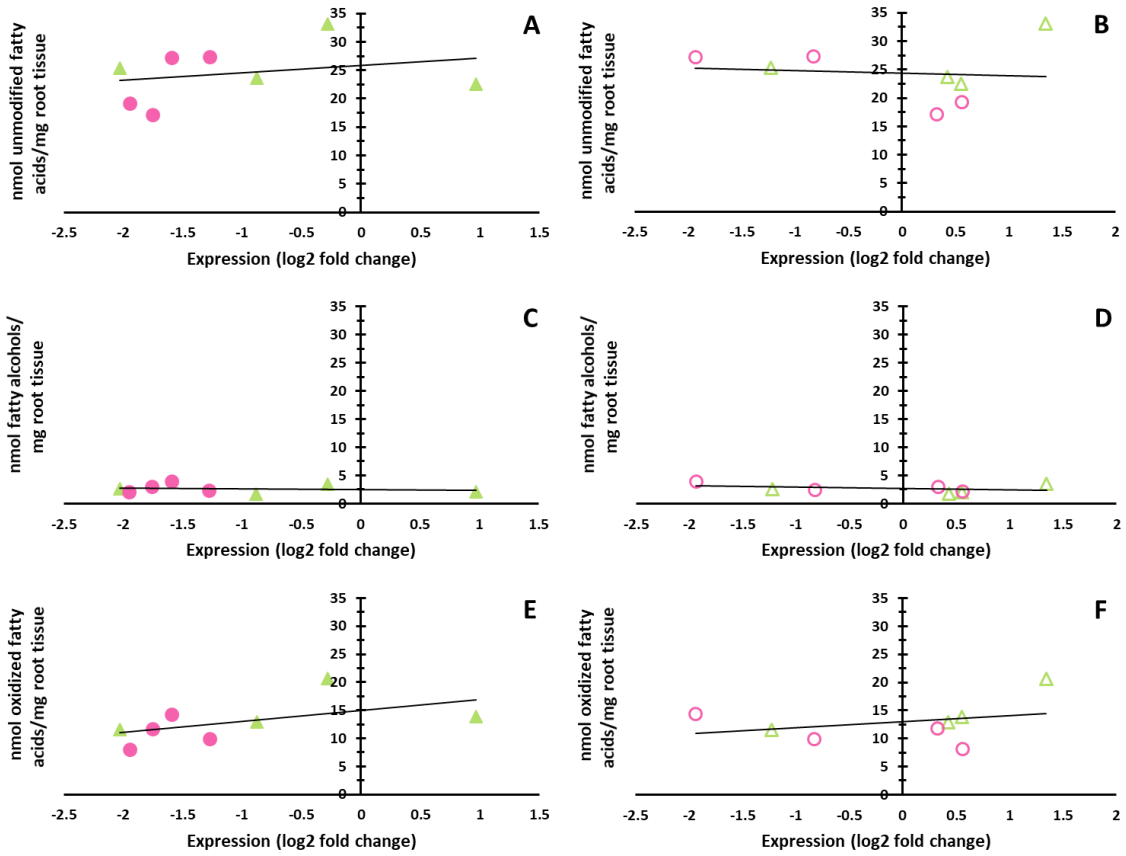


**Figure 3.6 Relationship between the expression of *GmCYP86A* genes and the three major compound classes that contribute to aliphatic suberin.** The expression of *GmCYP86A37* (left; closed symbols) and *GmCYP86A38* (right; symbols) is plotted relative to subsets of suberin monomers measured in the same tissues. Triangles represent controls, circles represent RNAi-knockdown lines. (A, B) unmodified fatty acids, (C, D) fatty alcohols, (E, F) oxidized fatty acids. Each data point represents an independent transformed hairy root line, including empty vector controls. Expression data are presented as $\log_2$ fold change, relative to the two reference genes (*cons6* and *cons7*). Trend lines are for visualization only. See Table 3.2 for statistics summary.

Next, in considering the predicted function of *CYP86A37* and *CYP86A38*, the ω-hydroxylated fatty acids were explored at the individual monomer level (i.e., specific individual chain lengths; Figure 3.7). *CYP86A38* expression showed a significant positive relationship with 18-hydroxy-oleic acid content (Table 3.3, Figure 3.7F). Furthermore, when only *cons7* was used as a reference gene in expression analysis, the strength of this relationship was greatly increased and *GmCYP86A37* expression also showed a significant positive relationship with 18-hydroxy-oleic acid content (data not shown).

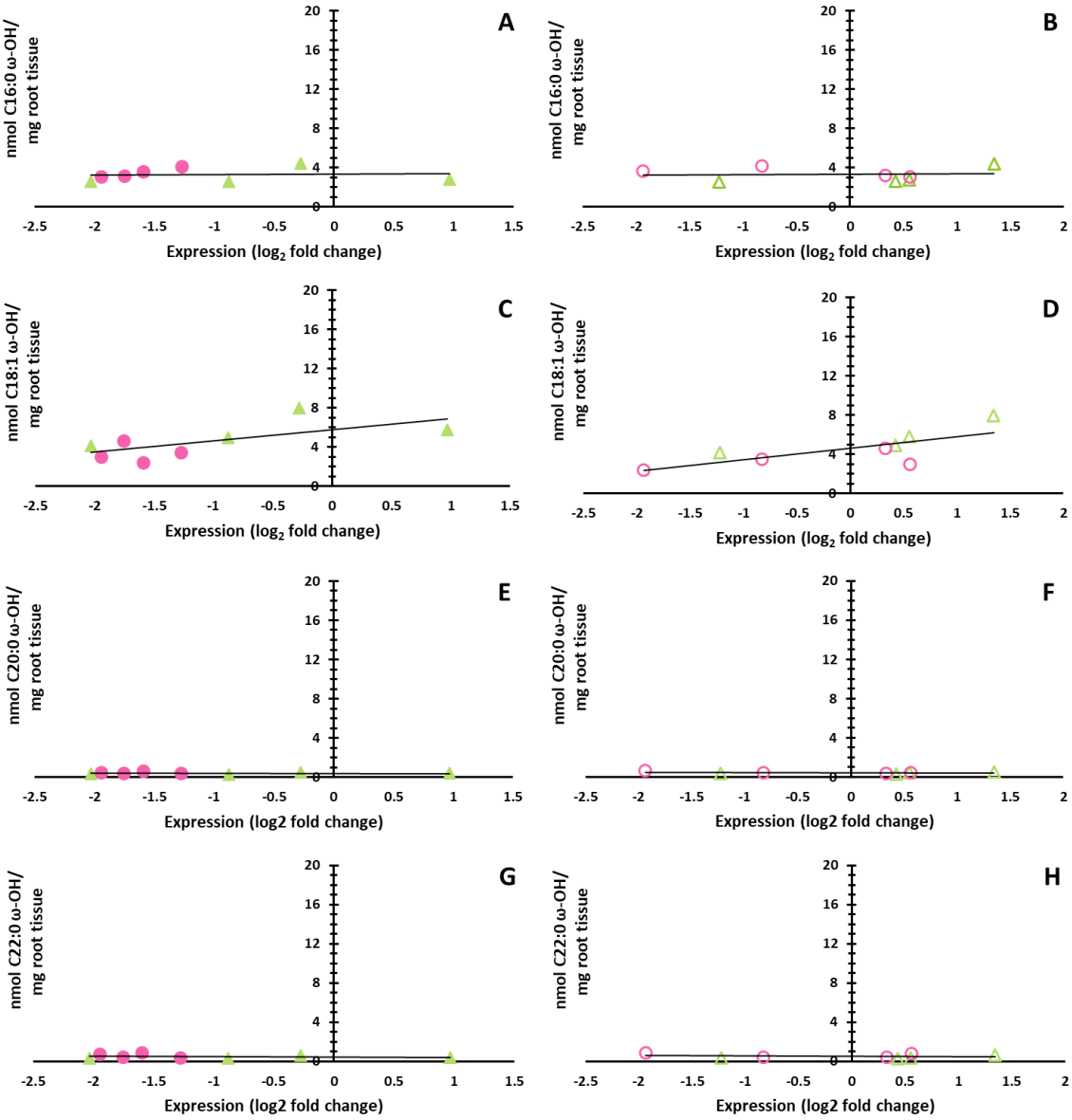


**Figure 3.7 Relationship between the expression of *GmCYP86A* genes and ω-hydroxy fatty acids from root suberin.** The expression of *GmCYP86A37* (left; closed symbols) and *GmCYP86A38* (right; open symbols) is plotted relative to ω-hydroxylated suberin monomers measured in the same tissues. Green triangles represent controls, pink circles represent RNAi-knockdown lines. (A, B) C16:0, (C, D) C18:1, (E, F) C20:0, and (G, H) C22:0. Each data point represents an independent transformed hairy root line, including empty vector controls Expression data are presented as $\log_2$ fold change, relative to the two reference genes (*cons6* and *cons7*). Trend lines are for visualization only. See Table 3.3 for statistics summary.

When pooled together, the *GmCYP86A37&38* knockdown lines showed a significant reduction in 18-hydroxy-oleic acid content (Figure 3.8; $t_{3.78} = 3.1041$, $p = 0.0389$), relative to empty vector control lines. By contrast, there were no significant differences in amount for any of the other ω-OH fatty acid monomer chain lengths in the RNA-knockdown lines relative to the control, (Figure 3.8).

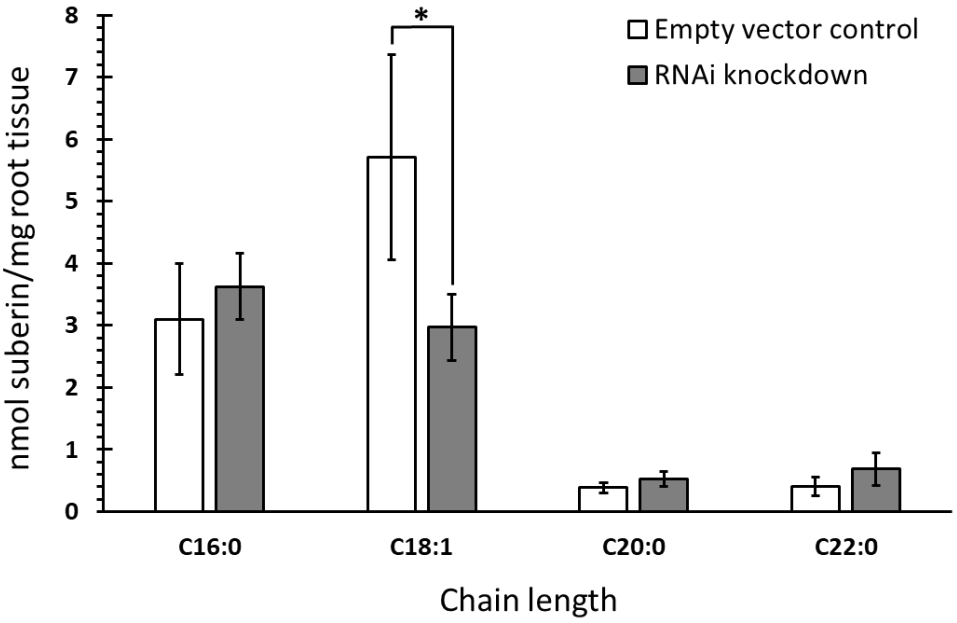


**Figure 3.8 Abundance of ω-hydroxylated suberin monomers of different chain lengths in *GmCYP86A* gene RNAi knockdown lines.** The abundance of different chain length ω-hydroxy fatty acids in hairy roots generated with an empty vector (control; open boxes, N = 4) or pHR37&38 RNAi knockdown vector (filled boxes, N = 3) was measured by GC-MS. For analysis, the four control lines and three pHR37&38 RNAi knockdown lines were combined (separately). The asterisk indicates a significantly higher 18-hydroxy-oleic acid content in control versus the RNAi knockdown lines ($t_{3.78} = 3.1041$, $p < 0.05$).

## 3.4 Aliphatic suberin content varies with developmental age but not cultivar

Aliphatic suberin analysis of three cultivars (OX760-6, Conrad, and Williams) across three developmental ages (tip, middle, top) revealed an increase in total aliphatic suberin content with increasing tissue age ($F_{1.77,10,62}$ = 6.74, $p$ = 0.03), but indicated no significant difference between cultivar ($F_{2,6}$ = 0.79, $p$ = 0.49; Figure 3.9A). When considering monomer subclasses, the unmodified fatty acids (tissue age: $F_{1.80,10,83}$ = 5.72, $p$ = 0.02; cultivar: $F_{2,6}$ = 0.07, $p$ = 0.93; Figure 3.9B) and oxidized fatty acids continued to follow this trend (tissue age: $F_{1.78,10,71}$ = 26.67, $p$ < 0.0001; cultivar: $F_{2,6}$ = 1.23, $p$ = 0.36; Figure 3.9C), where the fatty-alcohols (tissue age: $F_{1.73,10,39}$ = 2.33, $p$ = 0.15; cultivar: $F_{2,6}$ = 0.63, $p$ = 0.90; Figure 3.9D) showed no significant difference across either cultivar or developmental age.

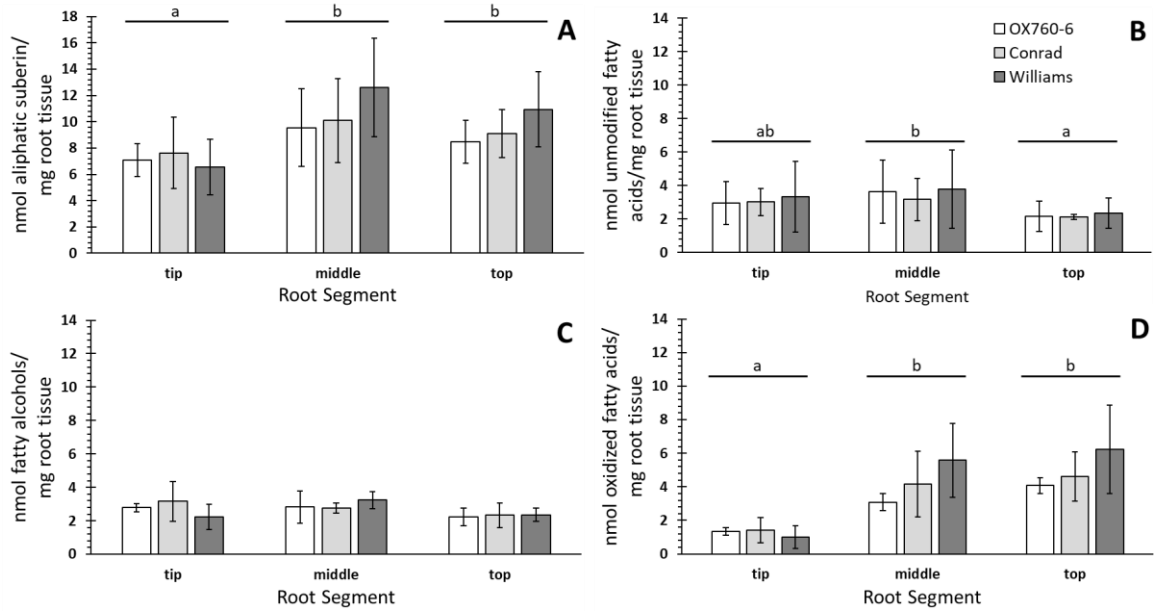


**Figure 3.9 Aliphatic suberin content in cultivars OX760-6, Conrad, and Williams across three developmental ages (determined by root segment; tip, middle, top).** Suberin abundance was determined for total aliphatics (A), unmodified fatty acids (B), fatty alcohols (C), and oxidized fatty acids (D) by GC-MS and is grouped by root segment, with different coloured bars representing the different cultivars. Bars represent means ± SD ($n$ = 3) and bars with the same letter within each subplot are not significantly different based on two-way RM-ANOVA followed by Tukey's HSD ($\alpha$ = 0.05).

To further examine the suberin profile of these three cultivars the abundance across root segments was summed, and the oxidized fatty acids were observed at the level of individual monomer (Figure 3.10). While no significant differences were observed between cultivars (Pillai's Trace = 1.3202, $F_{12,4}$ = 0.6473, $p$ = 0.7486), there was a trend of increasing abundance of total aliphatics (OX760-6 < Conrad < Williams) that appears to be driven by changes in abundance of the long chain length ω-hydroxylated monomers (C16:0 and C18:1).

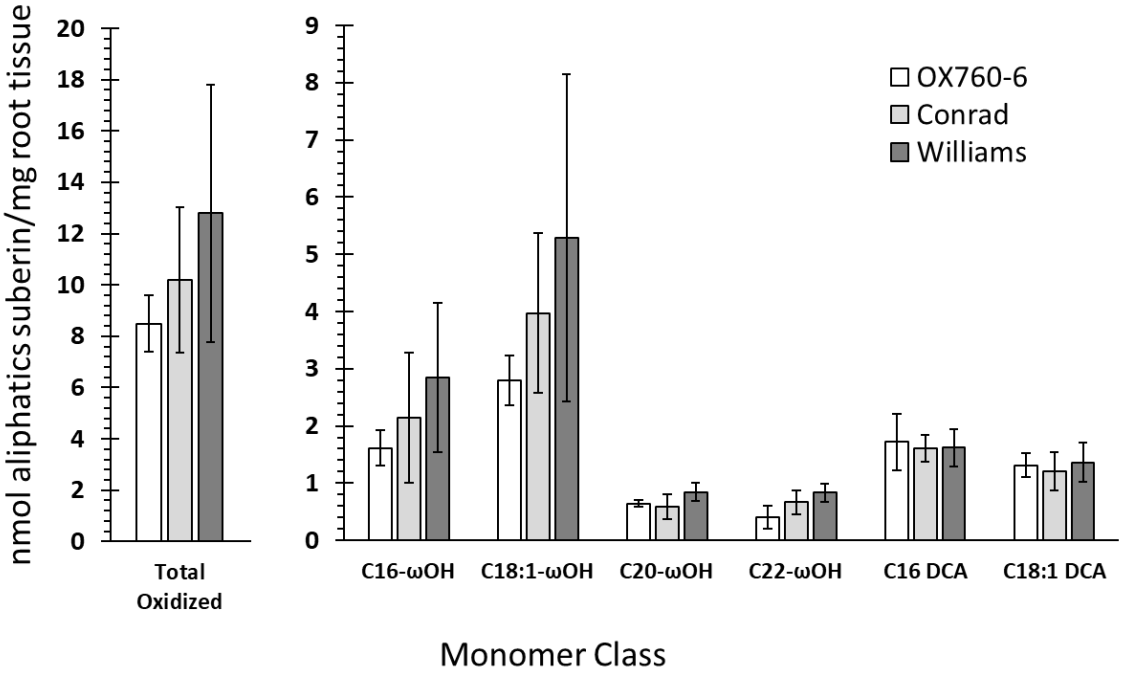


**Figure 3.10 Abundance of ω-hydroxylated suberin monomers of different chain lengths across cultivars.** Bars represent means ± standard deviation ($n$ = 3). No significant difference between cultivars was observed based on one-way MANOVA (Pillai's Trace = 1.3202, $F_{12,4}$ = 0.6473, $p$ = 0.7486).

## 3.5 Expression of CYP86A37 and CYP86A38 varies with developmental age but not cultivar

Similar to the aliphatic suberin profiles observed in this tissue, the expression of two key suberin biosynthesis genes (*CYP86A37* and *CYP86A38*) showed age-dependent but not cultivar-dependent expression patterns (Figure 3.11). Expression of these genes was significantly lower in the tip than the middle or top segments of the root tissue.

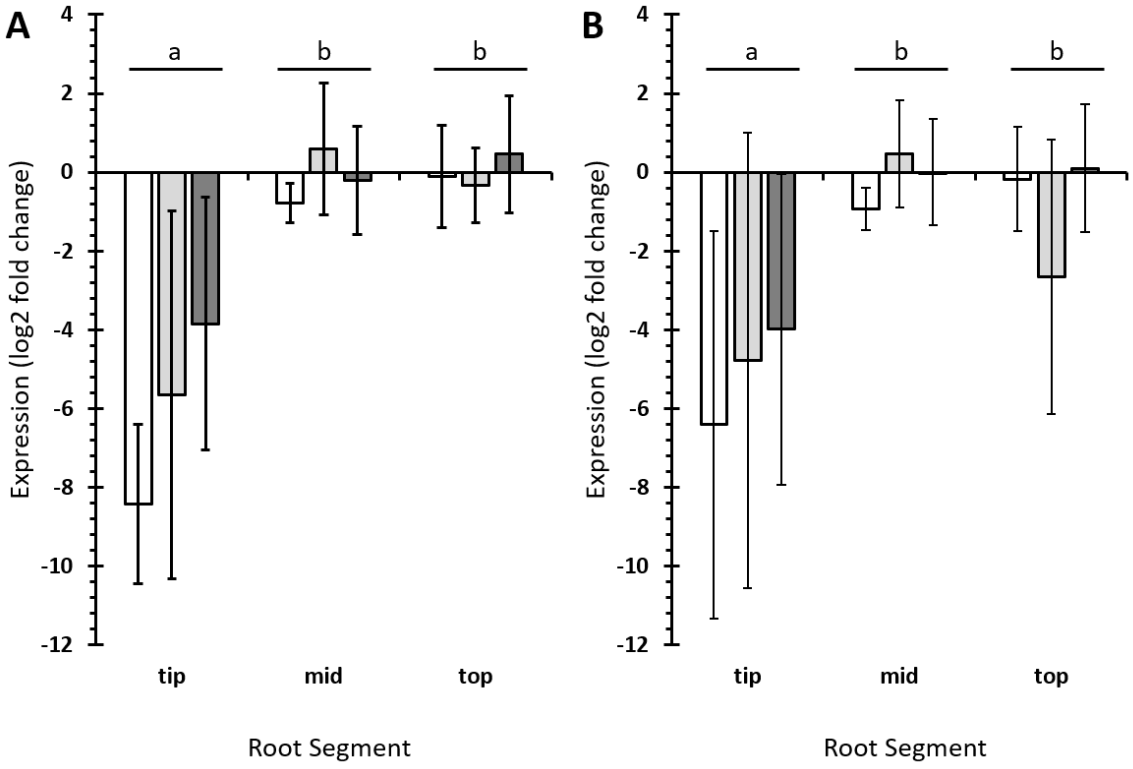


**Figure 3.11 Relative expression of *CYP86A37* and *CYP86A38* in cultivars OX760-6, Conrad, and Williams across three developmental ages (determined by root segment; tip, middle, top).** The relative expression of *CYP86A37* (A) and *CYP86A38* (B) was determined using the reference genes *cons4* and *cons7* and the "relative to none" feature of the ΔΔCq method and the Gene Study package in Bio-Rad CFX Manager™ (Software Version 3.1) as no single cultivar or root segment acts as a clear control. Values represent expression of means for each tissue type ± SD ($n$ = 3) after $\log_2$ transformation to allow for better visualization. Values with the same letter within each subplot are not significantly different based on two-way RM-ANOVA followed by Tukey's HSD (A: $F_{1.31,7.87} = 26.07$, $p < 0.001$; B: $F_{1.21,7.26} = 6.17$, $p = 0.04$).

## 3.6 The relationship between CYP86A37 and CYP86A38 expression and aliphatic suberin content

To assess the relationship between aliphatic suberin deposition and gene expression during development, all cultivars and developmental ages were plotted together. There is a significant positive relationship between gene expression for both *CYP86A37* and *CYP86A38* and total aliphatic suberin content (Table 3.4, Figure 3.12). To determine the impact of *CYP86A37* and *CYP86A38* expression on aliphatic suberin composition, the three major monomer classes were also observed separately. For the oxidized fatty acids, where there is a significant positive relationship between both *CYP86A37* and *CYP86A38* and oxidized suberin monomers (Table 3.5, Figure 3.13E,F). This relationship does not exist for either unmodified fatty acids (Table 3.5, Figure 3.13A,B) or fatty alcohols (Table 3.5, Figure 3.13C,D).

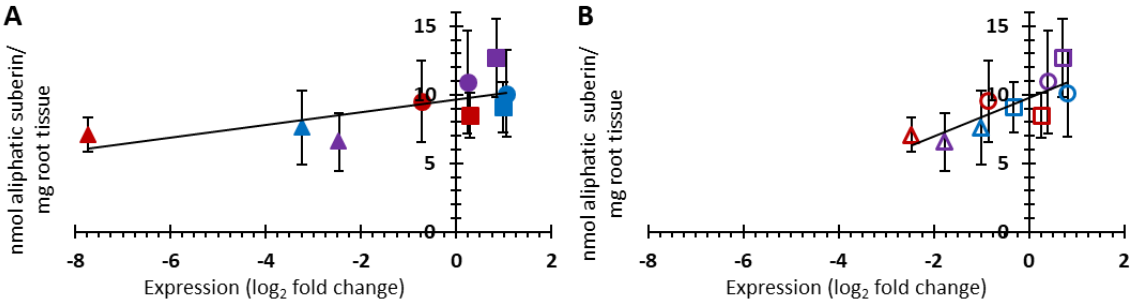


**Figure 3.12 Relationship between the expression of GmCYP86A genes and total aliphatic suberin.** The expression of *GmCYP86A37* (A) and *GmCYP86A38* (B) is plotted relative to total aliphatic suberin measured in the same tissues. Symbols represent root segments (triangles = tip, circles = middle, squares = top) and colours represent cultivar (red = OX760-6, blue = Conrad, and Purple = Williams). Each data point represents the expression value and mean suberin abundance across biological replicates ($n$ = 3). Error bars for suberin values = SD. Expression data are presented as $\log_2$ fold change, relative to the two reference genes (*cons4* and *cons7*). Trend lines are for visualization only. See Table 3.4 for statistics summary.

**Table 3.4 Summary of correlation analysis of CYP86A gene expression and total aliphatic suberin content across cultivars.**

| X- variable | Y-variable | Correlation Coefficient | 95% Confidence Interval | P-value |
|---|---|---|---|---|
| *CY986A37* expression | Total aliphatic suberin | 0.6822 | 0.0332, 0.9266 | **0.0429** |
| *CY986A38* expression | Total aliphatic suberin | 0.8160 | 0.3316, 0.9599 | **0.0073** |

**Table 3.5 Summary of correlation analysis of CYP86A gene expression and monomer subclass content across cultivars.**

| X- variable | Y-variable | Correlation Coefficient | 95% Confidence Interval | P-value |
|---|---|---|---|---|
| *CY986A37* expression | Unmodified fatty acids | -0.3695 | -0.8300, 0.3904 | 0.3277 |
| | Fatty alcohols | -0.3820 | -0.8344, 0.3781 | 0.3104 |
| | Oxidized fatty acids | 0.7333 | 0.1349, 0.9398 | **0.0246** |
| *CY986A38* expression | Unmodified fatty acids | -0.4618 | -0.8617, 0.2918 | 0.2108 |
| | Fatty alcohols | -0.1924 | -0.7595, 0.5408 | 0.6199 |
| | Oxidized fatty acids | 0.8439 | 0.4090, 0.9664 | **0.0042** |

**Table 3.6 Summary of correlation analysis of CYP86A gene expression and ω-hydroxylated monomer content across cultivars.**

| X- variable | Y-variable | Correlation Coefficient | 95% Confidence Interval | P-value |
|---|---|---|---|---|
| *CY986A37* expression | $C_{16:0}$-ωOH | 0.7906 | 0.2663, 0.9539 | **0.0112** |
| | $C_{18:1}$-ωOH | 0.7592 | 0.1918, 0.9462 | **0.0177** |
| | $C_{20:0}$-ωOH | 0.6551 | -0.0160, 0.9193 | 0.0555 |
| | $C_{22:0}$-ωOH | 0.7775 | 0.2343, 0.9507 | **0.0137** |
| *CY986A38* expression | $C_{16:0}$-ωOH | 0.8840 | 0.5326, 0.9755 | **0.0016** |
| | $C_{18:1}$-ωOH | 0.8565 | 0.4461, 0.9693 | **0.0032** |
| | $C_{20:0}$-ωOH | 0.7317 | 0.1315, 0.9394 | **0.0250** |
| | $C_{22:0}$-ωOH | 0.8702 | 0.4883, 0.9724 | **0.0023** |

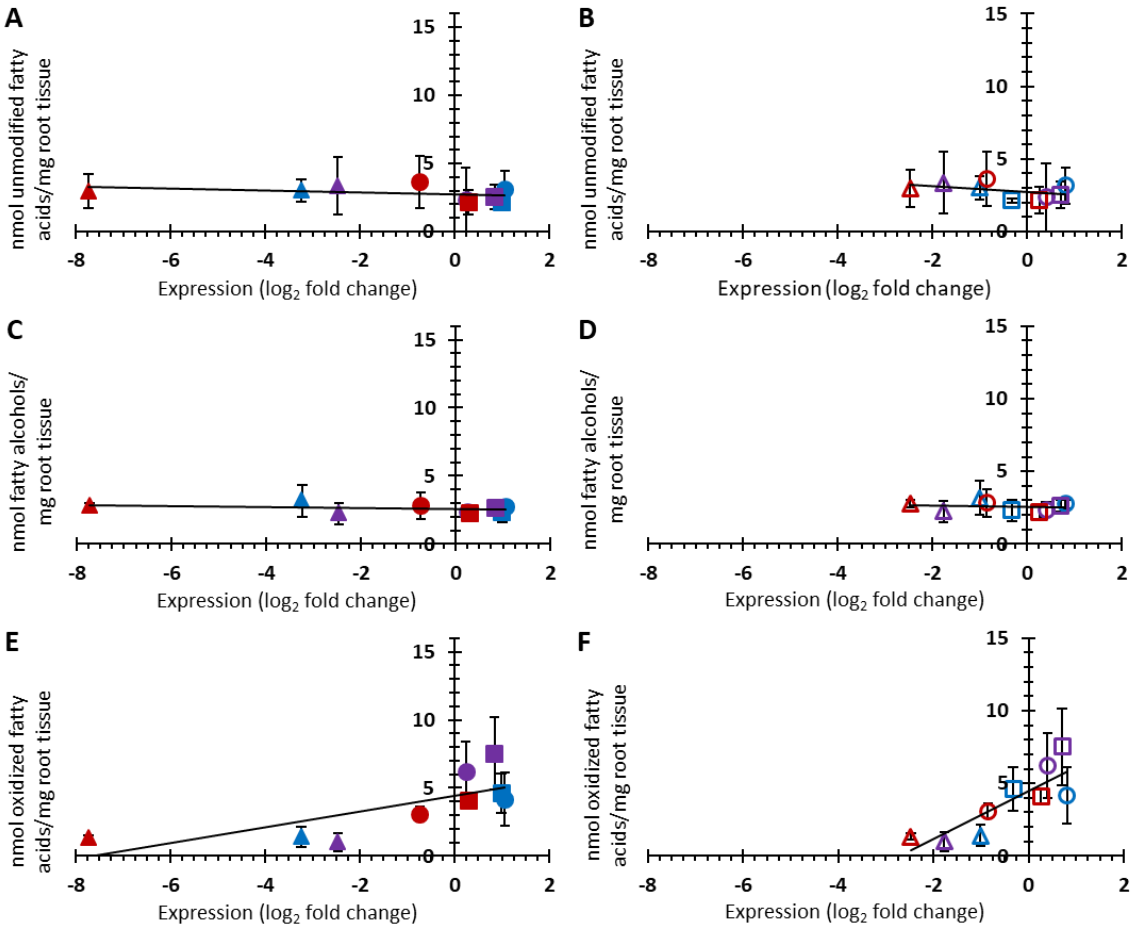


**Figure 3.13 Relationship between the expression of GmCYP86A genes and the three major compound classes that contribute to aliphatic suberin.** The expression of *GmCYP86A37* (left; closed symbols) and *GmCYP86A38* (right; symbols) is plotted relative to subsets of suberin monomers measured in the same tissues. Symbols represent root segments (triangles = tip, circles = middle, squares = top) and colours represent cultivar (red = OX760-6, blue = Conrad, and Purple = Williams). (A, B) unmodified fatty acids, (C, D) fatty alcohols, (E, F) oxidized fatty acids. Each data point represents the expression value and mean suberin abundance across biological replicates ($n$ = 3). Error bars for suberin values = SD. Expression data are presented as $\log_2$ fold change, relative to the two reference genes (*cons4* and *cons7*). Trend lines are for visualization only. See Table 3.4 for statistics summary.

As CYP86A37 and CYP86A38 are predicted to function as fatty acid ω-hydroxylases, the ω-hydroxylated fatty acids were further explored at the individual monomer level. A significant positive relationship exists between both *CYP86A37* and *CYP86A38* expression and ω-hydroxylated fatty acids regardless of chain length (with the exception of *CYP86A37* expression and $C_{20:0}$; Table 3.5, Figure 3.14). However, the strength of relationship (as indicated by the slope of the line) is not equal for all chain lengths (see Figure 3.14). In contrast to the ω-hydroxylated fatty acids, there is no significant relationship between *CYP86A37* or *CYP86A38* expression and the dicarboxylic acid monomers contributing to aliphatics suberin content (data not shown). For the strongest of the relationships, (16-hydroxy-palmitic acid and 18-hydroxy-oleic acid), each cultivar was plotted individually to see if the relationship between *CYP86A37* and *CYP86A38* expression and suberin content occurs independent of cultivar. A positive trend is clear for both 16-hydroxy-palmitic acid (Figure 3.15) and 18-hydroxy-oleic acid (Figure 3.16) in all three cultivars.

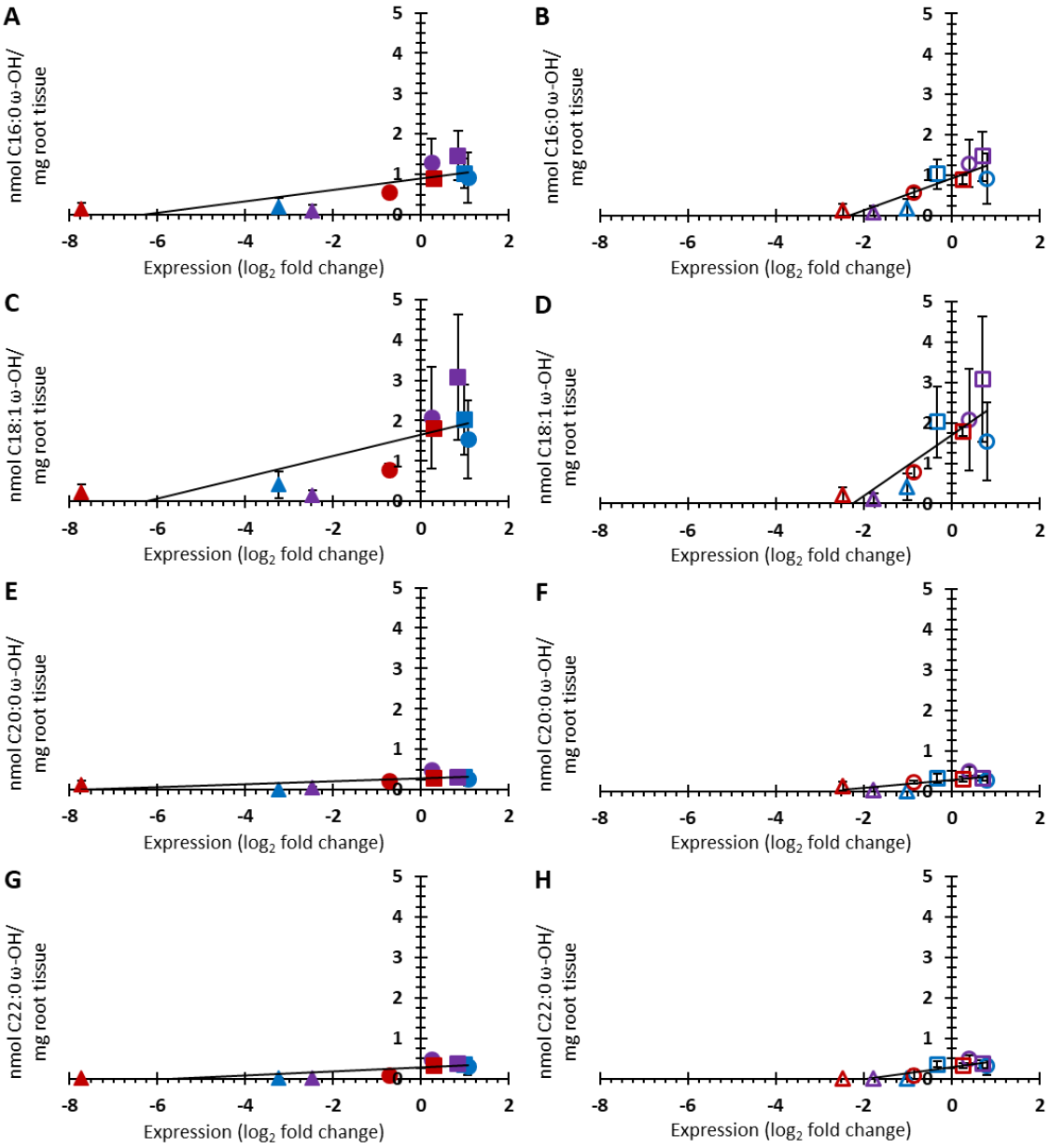


**Figure 3.14 Relationship between the expression of GmCYP86A genes and the ω-hydroxylated fatty acids that contribute to aliphatic suberin.** The expression of *GmCYP86A37* (left; closed symbols) and *GmCYP86A38* (right; symbols) is plotted relative to the abundance of specific suberin monomers measured in the same tissues. Symbols represent root segments (triangles = tip, circles = middle, squares = top) and colours represent cultivar (red = OX760-6, blue = Conrad, and Purple = Williams). (A, B) C16:0, (C, D) C18:1, (E, F) C20:0, (G, H) C22:0. Each data point represents the expression value and mean suberin abundance across biological replicates ($n$ = 3). Error bars for suberin values = SD. Expression data are presented as $\log_2$ fold change, relative to the two reference genes (*cons4* and *cons7*). Trend lines are for visualization only. See Table 3.4 for statistics summary.

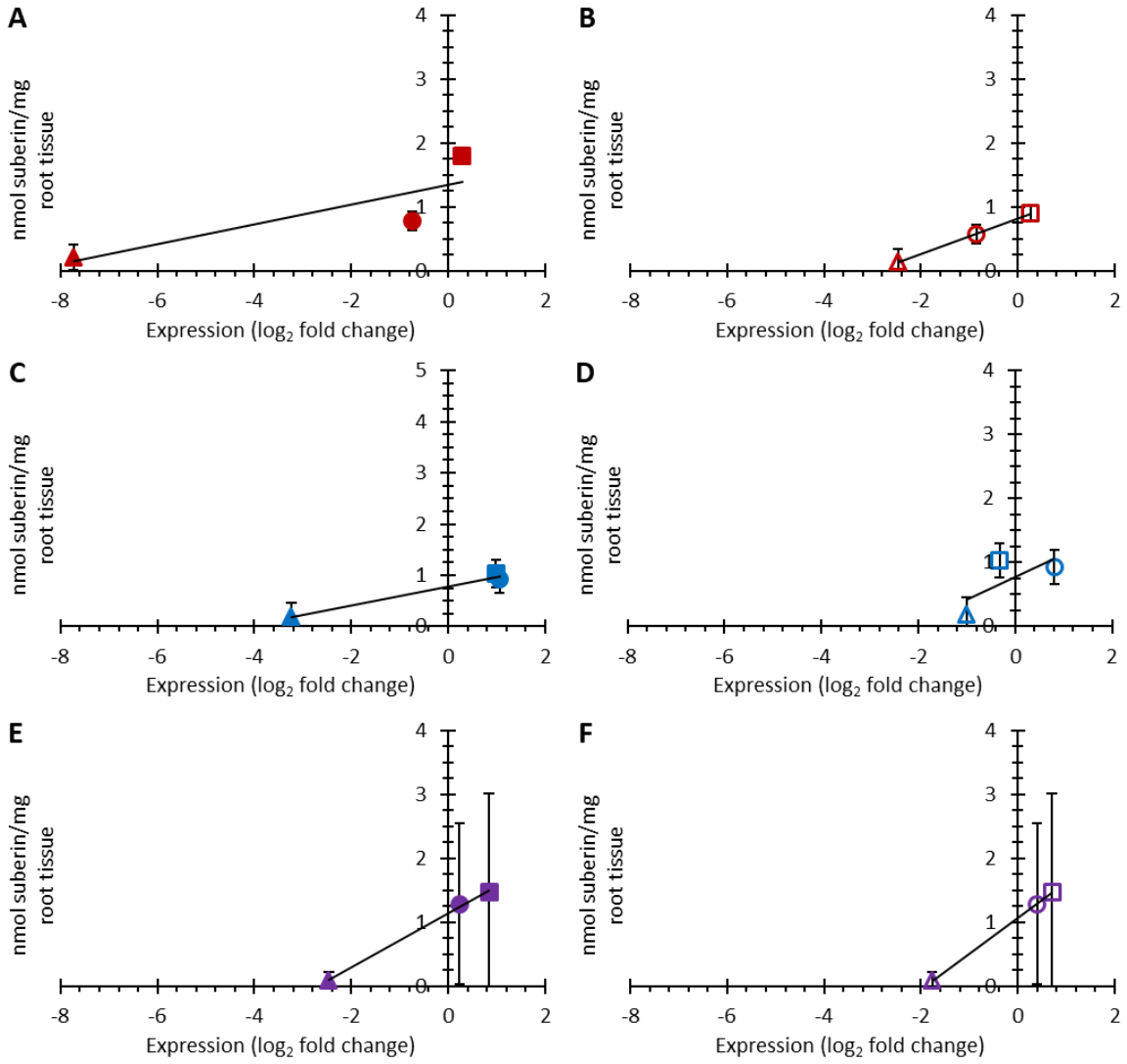


**Figure 3.15 Relationship between the expression of GmCYP86A genes and 16-hydroxy-palmitic acid.** The expression of *GmCYP86A37* (left; closed symbols) and *GmCYP86A38* (right; symbols) is plotted relative to the abundance of specific suberin monomers measured in the same tissues. Symbols represent root segments (triangles = tip, circles = middle, squares = top). (A, B; red) OX760-6, (C, D; blue) Conrad, (E, F; purple) Williams. Each data point represents the expression value and mean suberin abundance across biological replicates ($n$ = 3). Error bars for suberin values = SD. Expression data are presented as $\log_2$ fold change, relative to the two reference genes (*cons4* and *cons7*). Trend lines are for visualization only.

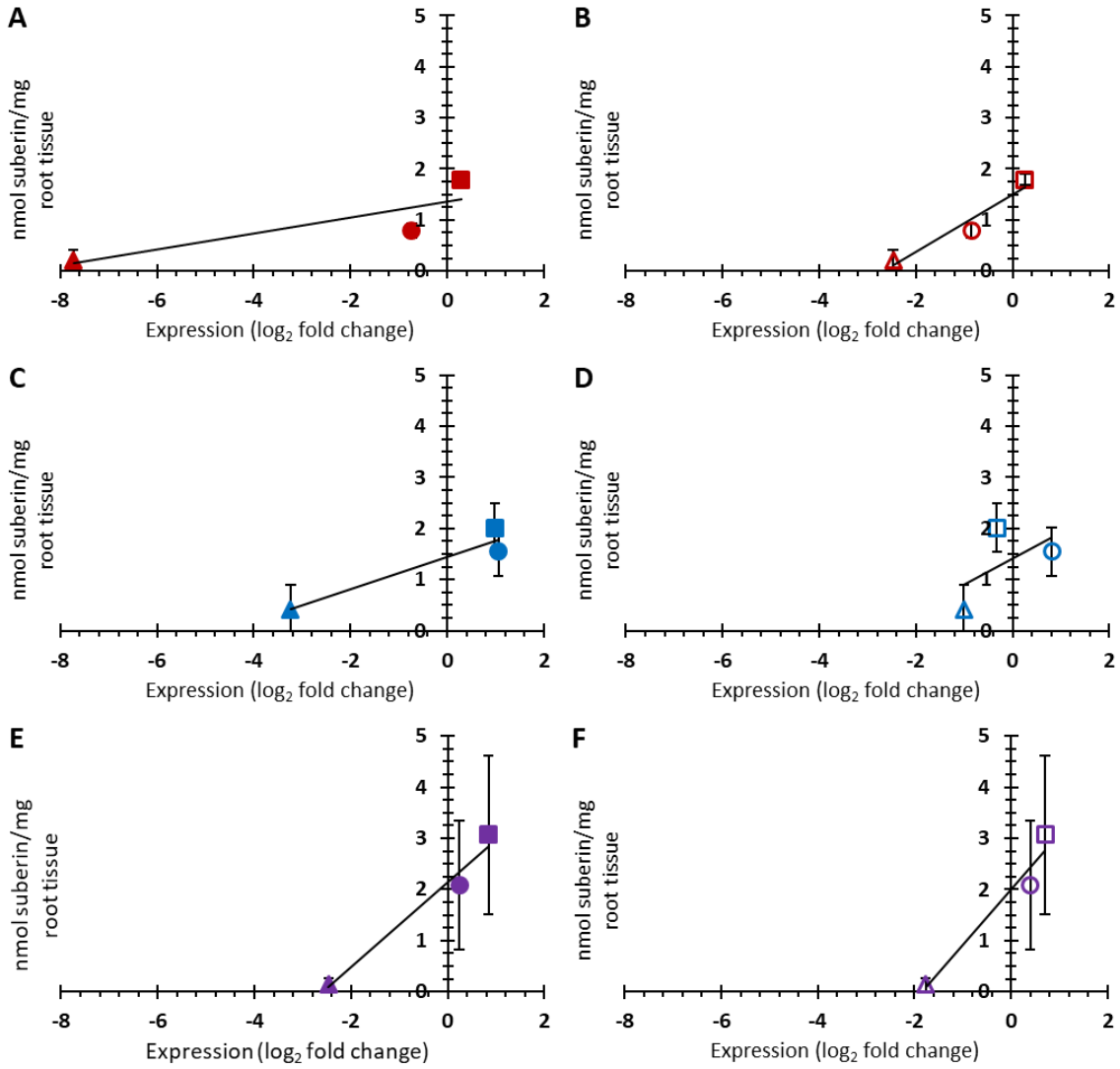


**Figure 3.16 Relationship between the expression of GmCYP86A genes and 18-hydroxy-oleic acid.** The expression of *GmCYP86A37* (left; closed symbols) and *GmCYP86A38* (right; symbols) is plotted relative to the abundance of specific suberin monomers measured in the same tissues. Symbols represent root segments (triangles = tip, circles = middle, squares = top). (A, B; red) OX760-6, (C, D; blue) Conrad, (E, F; purple) Williams. Each data point represents the expression value and mean suberin abundance across biological replicates ($n$ = 3). Error bars for suberin values = SD. Expression data are presented as $\log_2$ fold change, relative to the two reference genes (*cons4* and *cons7*). Trend lines are for visualization only.

# 4 DISCUSSION

The developmental deposition of suberin in the root tissue of plants is well-established. While the general process is understood, our depth of understanding of specific details is still lacking, due in part to the complexity of the suberin polymer itself. Suberin biosynthesis is hypothesized to include specific metabolic pathways, such as the *de novo* biosynthesis and subsequent modification of fatty acids. However, many details of the more specialized (i.e., suberin specific) pathways, including the enzymes involved, their regulation and the overall temporal regulation of the pathways are yet to be elucidated. Suberin plays a role in the response of plants to many environmental challenges, including drought and pathogen resistance, which are becoming increasingly important in a changing climate. Without further development of our understanding of the suberization process, the remaining gaps in knowledge leave us ill equipped to best understand and manipulate suberin biosynthesis, and thus develop cultivars that are better equipped to survive environmental challenges. This thesis addresses part of this knowledge gap by providing data in support of the function of *CYP86A37* and *CYP86A38* genes as fatty acid ω-hydroxylases.

Building on the link between CYP86A37 and CYP86A38 gene expression and aliphatic suberin deposition in a hairy root model system, I explored the relationship between the expression of these two genes and aliphatic suberin deposition during root development of soybean seedlings. For this, I measured the CYP86A37 and CYP86A38 gene expression and suberin deposition in seedlings of three soybean cultivars that have been shown to differ in the amount of suberin they deposit in their roots (OX760-6, Conrad, and Williams; Thomas et al. 2007). From this, I established the relationship between aliphatic suberin deposition and gene expression *in planta*.

## 4.1 The soybean genome contains 14 putative fatty acid ω-hydroxylase genes

In plants, fatty acid omega-hydroxylases (FAωHs) are grouped into four main subfamilies; CYP86As, CYP86Bs, CYP94As, and CYP704Bs, of which the first three are known to be involved in cutin and aliphatic suberin monomer biosynthesis in some species.

Phylogenetic analysis of the soybean genome for suberin associated FAωHs revealed 14 putative FAωH genes in soybean; six *CYP86A*s, three *CYP86B*s, and five *CYP94A*s (Figure 3.1). As the soybean genome is considered to be a polypaleoploid (Schmutz et al. 2010), it is likely that there is some redundancy in terms of the functional capacity of these genes, making the tissue specific expression pattern as indicated by RNA Seq data unsurprising (see Appendix C; Grant et al. 2010). By combining the phylogenetic relationship of these 14 putative FAωHs genes to suberin associated homologues (Le Bouquin et al. 2001; Höfer et al. 2008; Compagnon et al. 2009), and their gene expression profiles (see Appendix C; Figure 3.3A,B), I have identified five genes as candidates for a role in suberin biosynthesis; *CYP86A37*, *CYP86A38*, *CYP86B9*, *CYP94A17*, and *CYP94A19*. As long chain (esp. 18:1) ω-OH and dioic fatty acids are the predominant monomers in soybean aliphatic suberin, I focused further exploration on genes in the subfamily responsible for long chain ω-OH and dioic fatty acid biosynthesis, namely CYP86As (Höfer et al. 2008). This resulted in two candidates: *CYP86A37* and *CYP86A38*.

## 4.2 Gene function analysis using a hairy root model system

While working with soybean offers many genetic advantages including a sequenced genome (Schmutz et al. 2010) and databases like Soybase.org (Grant et al. 2010), it is also challenging in that soybean is not easily transformed using conventional techniques such as *Rhizobium radiobacter*-mediated and ballistic transformation (Kereszt et al. 2007; Cao et al. 2009; Li et al. 2010; Mangena et al. 2017). The soybean genome is generally well annotated; however, functional characterization of putative gene annotations requires direct manipulative experimentation, and a system in which this can occur. For root specific genes *Rhizobium rhizogenes*-mediated transformation, which yields genetically modified hairy roots, offers a good alternative.

Overall, the anatomy of hairy roots is similar to that of soil-grown roots, with two notable differences: hairy roots sometimes have only three xylem poles (rather than the four xylem poles typical of soil-grown roots) and the cortex appeared less compact than soil-grown roots (Sharma 2012). However, what is key is that the tissue specific deposition of suberin in endodermal and epidermal root cells is the same between hairy roots and soil

grown roots (Sharma 2012). For study of the suberin specific gene candidates *CYP86A37* and *CYP86A38*, I established that their gene expression patterns in, and the suberin composition of hairy roots matched that observed in soil-grown roots which allowed me to use the hairy roots system as a model to proceed with a reverse genetics approach in the study of *CYP86A37* and *CYP86A38*.

## 4.3 *CYP86A37* and *CYP86A38* likely encode functional fatty acid ω-hydroxylases

Since the function of a CYP86A is ω-hydroxylation of fatty acids (Durst and Nelson 1995; Rupasinghe et al. 2007), I examined the monomers of this class more closely. This revealed a positive correlation between expression *CYP86A37* or *CYP86A38* and the amount of ω-hydroxy-fatty acids monomers in hairy root knock-down tissue that indicates a potential relationship between gene expression and ω-hydroxylated monomers. Specifically, reduction in *CYP86A37* and *CYP86A38* expression resulted in downstream reduction of 18-hydroxy-oleic acid content in the roots, while the ω-hydroxylated monomers of other chain lengths remained unaffected (Figure 3.7C-J). This change at the level of individual monomer strongly suggests that *CYP86A37* and *CYP86A38* encode functional FAωHs responsible for the biosynthesis of 18-hydroxy-oleic acid, which is consistent with previous literature that suggests a high substrate specificity for shorter chain fatty acids for this subfamily of enzymes (Duan and Schuler 2005). For example, ω-hydroxylases in Arabidopsis are chain length specific, where *AtCYP86A1* mutants show a reduction in only long chain ($C_{16}$ and $C_{18}$) ω-OHs (Höfer et al. 2008) and *AtCYP86B* mutants show a reduction in only long chain ($C_{22}$ and $C_{24}$) ω-OHs (Compagnon et al. 2009). That being said, it is important to note that the resolution of my experiment does not allow for confirmation of substrate specificity of these two genes.

## 4.4 Synthesis of oxidized fatty acids in soybean requires more than *CYP86A37* and *CYP86A38*

In examining the phenotypic response to changes in gene expression introduced by knockdown constructs for *CYP86A37* and *CYP86A38*, a net change in aliphatic suberin is

not immediately evident, as there is no significant correlation between expression of these two genes and total aliphatic suberin content (Figure 3.5). This absence of a relationship between expression of *CYP86A37* or *CYP86A38* and total aliphatic suberin content was surprising, especially when compared to similar experiments in Arabidopsis and potato (Höfer et al. 2008; Serra et al. 2009b). When comparing aliphatic suberin content between wild-type and the *horst* mutant of Arabidopsis, there was a significant reduction in total aliphatic suberin content in the mutant (Höfer et al. 2008). Similarly, in potato, a reduction in total aliphatic suberin was also observed in *StCYP86A33*-knockdown mutants when compared to wildtype (Serra et al. 2009b). As the two genes characterized in potato and the Arabidopsis knock down mutants (*StCYP86A33* and *AtCYP86A1*) are likely orthologues to *CYP86A37* and *CYP86A38*, it was not unreasonable to expect a similar reduction in aliphatic suberin content when knocking down these soybean genes. Previous literature suggested reduction in oxidized fatty acids when *CYP86A* expression was reduced (Höfer et al. 2008; Serra et al. 2009; Figure 3.6).

While unexpected, this result can partly be explained by considering the difference between *knockdown* and *knockout* of a gene. In both the *horst* and *StCYP86A33* mutants, the target gene was knocked out resulting in no measurable transcript from which to synthesize enzyme. By contrast, my *CYP86A37* and *CYP86A38* knockdown lines still had some transcript remaining available and this was likely sufficient for synthesis of a low level of enzyme. In addition, suberin biosynthesis and deposition is a complex process, and it is possible that other genes involved may compensate for the reduction in *CYP86A37* and *CYP86A38* expression, thus resulting in a lack of impact on total aliphatic suberin. For example, increased production ω-hydroxylated fatty acids of other chain lengths could occur, meaning the composition but not the amount of aliphatic suberin deposited would be altered. The lack of impact on total aliphatic suberin levels in *CYP86A37* and *CYP86A38* knockdown lines also suggests that other FAωHs are likely involved in suberin biosynthesis in soybean. As indicated above, the soybean genome contains at least 12 other putative FAωHs, including three that are expressed in root tissue (*CYP86B9*, *CYP94A17*, and *CYP94A19*; see Appendix C). Based on phylogenetic evidence, one might speculate that these are involved in ω-hydroxylation of longer chain fatty acids (*CYP86B9*) or formation of DCAs (*CYP94A17*, and *CYP94A19*) in suberin biosynthesis, in addition to

*CYP86A37* and *CYP86A38*. However, there is also evidence that CYP86B and CYP94A enzymes do not have as profound an impact on suberin biosynthesis as CYP86As. For example, in contrast to the *horst* phenotype, the *AtCYP86B1* knock out mutant *ralph* shows a reduction in specific monomers, $C_{22}$ and $C_{24}$ ω-OHs, without subsequent reduction in total aliphatic suberin content (Ranathunge and Schreiber 2011). Furthermore, monomers produced by CYP86As (i.e long chain length ω-OHs) have been implicated in the cross-linking of the two suberin domains (a key step in suberin biosynthesis) whereas longer chain length monomers have yet to be linked to this role (Graça et al. 2015). Regardless of which of these reasonings for the differences observed between my experiment and previous ones, further experimental verification will be required to confirm.

## 4.5 Gene expression and suberin deposition patterns across cultivars reveal unexpected patterns

With the link between expression of *CYP86A37* and *CYP86A38* and aliphatic suberin deposition, more specifically the positive correlation between abundance of the predominant monomer 18-hydroxy-oleic acid and *CYP86A37*/*CYP86A38* expression, established through RNAi-knockdown in soybean hairy roots, the next logical step was to investigate whether this relationship persists *in planta* over the course of normal growth and development. To achieve an experimental system that could explore both normal growth and development and differences in suberin abundance, cultivars that have been shown to vary in the amount of suberin normally deposited in their roots (Thomas et al. 2007) were grown from seed in vermiculite to generate roots for analysis.

With this system, my first approach was to observe gene expression in whole root tissue of three soybean cultivars previously shown to different in the amount of aliphatic suberin deposited in their root tissue, namely OX760-6, Conrad, and Williams (Thomas et al. 2007; data not shown). As 18-hydroxy-oleic acid is the predominant monomer in soybean aliphatic suberin, I predicted that the expression level of *CYP86A37* and *CYP86A38* would be positively correlated with previously recorded aliphatic suberin levels. The pattern of expression from whole root analysis was not as expected; the expression level of *CYP86A37* and *CYP86A38* was not positively correlated with previously recorded aliphatic suberin levels. However, when considering these results

more carefully, I concluded that using bulk root tissue may not have been a good representative sample to describe gene expression in the roots, as the suberin phenotype is known to vary along the developmental axis (Thomas et al. 2007). Therefore, to explore the relationship between developmental suberin deposition and gene expression further, an additional experiment was designed, in which roots were segmented along a developmental root axis prior to gene expression and suberin analyses (see Chapter 2.7)

Gene expression analysis of the developmentally segmented root tissue revealed a similar pattern of expression for all three cultivars across the three developmental time points I considered. In brief, the tip of the root showed the lowest expression of *CYP86A37* and *CYP86A38* compared to the middle and top root segments (Figure 3.11). This indicated that, while there was a trend toward increased expression along a developmental axis, the expected differences in gene expression levels between cultivars were absent. Thomas et al. (2007) observed the highest level of suberin deposited in the cultivar Conrad, followed by Williams, then OX760-6. However, in my experiment, Williams shows the highest abundance of oxidized fatty acid monomers, followed by Conrad, then OX760-6. Considering the trend between these two experiments was not the same, and the magnitude of differences in aliphatic suberin deposition was observed to be much lower in this thesis, it will be important to confirm whether the trend in aliphatic suberin deposition across cultivars is indeed a persistent phenotype.

## 4.6 The relationship between *CYP86A37* and *CYP86A38* expression and aliphatic suberin deposition persists during normal growth and development

In contrast to the hairy root RNAi knockdown experiment, when relating expression of *CYP86A37* and *CYP86A38* to aliphatic suberin deposition in soil-grown plants, both genes showed a significant positive correlation with total aliphatic suberin content (Figure 3.12). This suggested that, while the expected differences in suberin deposition between cultivars were not observed, the relationship between *CYP86A37* and *CYP86A38* expression and aliphatic suberin deposition persisted during normal growth and development. In examining this association more closely, it mirrored the response in the hairy root RNAi knockdown lines, where the relationship between gene expression and

suberin deposition was again driven by changes in the abundance of oxidized fatty acids (Figure 3.13). However, the similarity between the two experiments didn't extend to the patterns observed with specific chain lengths of suberin monomers. That is, with the RNAi knockdown lines the observed reduction in oxidized fatty acids could be attributed solely to differences in 18-hydroxy-oleic acid but none of the other oxidized fatty acid monomers. In contrast, a positive relationship between expression of *CYP86A37* and *CYP86A38* and ω-hydroxylated monomers in general was observed for all chain lengths detected (with the exception of *CYP86A37* and 20-hydroxy-eicosanoic acid). So, where does this leave the role of CYP86A37 and CYP86A38 in aliphatic suberin deposition?

Both the hairy root knockdown lines and whole plant developmental studies demonstrated that *CYP86A37* and *CYP86A38* likely encode functional FAωHs responsible for the biosynthesis of 18-hydroxy-oleic acid. While these two genes show a significant correlation with ω-hydroxylated monomers of all four observed chain lengths, it is important to also consider whether these changes are biologically relevant. Over the same changes in gene expression, the magnitude of the change in monomer context is not the same across all chain lengths. For the long-chain monomers the change in monomer content is much greater than the change for the very long-chain monomers. From this perspective, the relationship between gene expression and aliphatic suberin deposition can be considered stronger for the two long-chain length ω-hydroxylated monomers, 16-hydroxy-palmitic acid and 18-hydroxy-oleic acid. than for the very long-chain length monomers. This indicates that CYP86A37 and CYP86A38 likely prefer long-chain substrates; however, as noted earlier, this study does not offer the resolution to confirm this substrate specificity. To be definitive in assigning substrate specificity, recombinant protein expression and detailed enzyme kinetic analysis is required.

## 4.7 Does the capacity for phenotypic improvement of suberin deposition exist?

To have the capacity for phenotypic improvement, there must exist genetic diversity for the genes that are responsible for the phenotype. In RNAi knockdown *CYP86A37* and *CYP86A38* lines, a reduction in 18-hydroxy-oleic acid monomers in root aliphatic suberin correlated with reduced gene expression, thereby altering suberin composition (Figure 3.8). Therefore, altering the expression of these two genes impacted the suberin phenotype. In

addition, while the gene expression for *CYP86A37* and *CYP86A38* did not differ between three cultivars with differing levels of total root suberin, there were differences in expression between developmental stages (Figure 3.11). That is, the differences in *CYP86A37* and *CYP86A38* expression along the root axis were mirrored by differences in suberin deposition, or phenotype (Figure 3.12). It is possible that the relationship between *CYP86A37* and *CYP86A38* gene expression and suberin deposition across cultivars had too small of an effect size to be detected in my experiments. Currently, while differences in monomer deposition were visually apparent across the three cultivars, they were not statistically significant. Given that suberin deposition is likely a multi-gene, quantitative trait (Thomas et al. 2007), it is possible that the contribution of *CYP86A37* and *CYP86A38* to the suberin phenotype of a given cultivar is small, as the genetic architecture of more complex traits (like suberin deposition) is often comprised of many loci with small effect sizes (Cobb et al. 2013). Accordingly, power analysis (R package pwr, function pwr.anova.test) suggested that to observe a small effect size (using package function cohen.ES) as a true positive in 80% of replicate experiments and with the experimental arrangement used herein would require a sample size of $n = 322$, which is not only an unfeasible number of replications, it is much greater than the sample sized used ($n = 3$). A sample size of this magnitude would reduce the amount of noise relative to the current data set and may turn subtle differences between cultivars into significant trends. While the additional information of whether or not the differences in suberin deposition between cultivars are significant may not seem biologically relevant if the effect size is small, it is important to remember that the compositional make up of suberin can be just as important to the phenotypic properties as total abundance (Schreiber et al. 2005b). This means a small shift in the compositional nature of suberin, driven by changes in *CPY86A37* and *CYP86A38* expression, has the potential to have a real impact on phenotypic properties and therefore the development of stress resistant cultivars of soybean.

The concept of small differences having large biological relevance can also be applied to the gene expression data collected across cultivars. Again, while no significant trend was observed across cultivars, this can potentially be explained by the nature of the experimental design. In soybean root tissue, suberin is deposited in only two specialized tissue layers in the root; the endodermis and the epidermis. Therefore, only the cells found

in these two layers would be expressing *CYP86A37* and *CYP86A38*. This means that in the tissue used for RNA extraction and subsequent qPCR analysis of *CYP86A37* and *CYP86A38* expression would have a low relative abundance of the two transcripts of interest. Given the trend that the lower the transcript abundance the higher the variation in amplification (Bustin and Nolan 2004), it is unsurprising that expression data from the three cultivars shows high variability (Figure 3.11). In the future there are several different strategies that could be used to minimize this variability including preparing cDNA from mRNA rather than total RNA (Bustin and Nolan 2004), isolating epidermal and endodermal layers prior to extraction, or switching to a more sensitive method of amplification like droplet digital PCR.

## 4.8 What else contributes to the suberin phenotype?

All soybean cultivars have the ability to produce suberin in their roots. What differs between cultivars is the abundance and/or compositional nature of aliphatic suberin that is deposited. Whether considering the subtle differences between cultivars observed in this thesis or the more pronounced differences observed by Thomas (2006), these changes in suberin phenotype between cultivars offer the opportunity to target a specific phenotype in developing crops resistant to challenging environments. While *CYP86A37* and *CYP86A38* expression are likely to be at least in part responsible for the variation in suberin phenotypes, since suberin deposition is a quantitative trait, not all of the variation in suberin deposition can be explained by changes in expression of these two genes. Instead, suberin biosynthesis and deposition requires additional biosynthesis genes as well as regulation of biosynthesis by transcription factors. During RNAi knockdown of *CYP86A37* and *CYP86A38*, the only notable change in monomer abundance was seen for a single suberin monomer (18-hydroxy oleic acid) and not total suberin content. This suggested that *CYP86A37* and *CYP86A38* play a role in composition, but not overall deposition of suberin. To validate this result, further experimentation involving genetic manipulation *CYP86A37* and *CYP86A38*, as well as the other putative FAωH genes in soybean (*GmCYP86B9, GmCYP94A17* and *GmCYP94A19*) would be required, which is beyond the scope of the techniques used in this experiment.

As biosynthesis of oxidized fatty acids by cytochrome P450 genes is crucial to the formation of the suberin polymer, it is logical to consider these genes when considering key genes for targeting a specific suberin phenotype. However, it is also possible that flux through this part of the suberin biosynthesis is regulated further upstream and may differ between cultivars. This means that in addition to the function and prevalence of these enzymes it is also important to consider both the regulation and the substrate availability for the synthesis of oxidized fatty acid monomers. As regulation of developmental suberin deposition is still poorly understood, it will be difficult to determine transcription factors critical to developmental suberin biosynthesis in soybean. However, future exploration of substrate availability and flux through the fatty acid biosynthesis pathway may provide a more straightforward approach. For example, to determine whether substrate availability differs across cultivars or suberin phenotypes, primary metabolism could be measured using a metabolomics approach (Schauer and Fernie 2006).

It would also be interesting to considering the epigenetic contribution to the aliphatic suberin phenotype. As the epigenome can be thought of as a combination of genetics plus the environment, it is clear that epigenetic factors should not be ignored when considering the impact of the environment on crop success. Changes in the epigenome can result in a plant "remembering" which stressors it has previously experienced (Chinnusamy and Zhu 2009; Kinoshita and Seki 2014; Bilichak and Kovalchuk 2016). For example, drought stress has been shown to trigger histone modification (Kinoshita and Seki 2014; Kim et al. 2015), whereas temperature and pathogen attack have contributed to the generation of differentially methylated regions DMRs including *defense priming* (e.g. hypomethylation of resistance genes making them more readily available for transcriptional activation; Feil and Fraga 2012; Kinoshita and Seki 2014; Espinas et al. 2016). Alteration of the epigenome by the environment has the potential to alter the suberin phenotype. For example, in potato, *StNAC103* knockdown results in an increase of suberin aliphatic accumulation (Verdaguer et al. 2016), which means this gene offers a target for hypermethylation that would result in increased suberin deposition. Similarly, in Arabidopsis, *dewax* mutants show an increase in deposition of cuticular wax (Kim et al. 2018). It is not unreasonable to hypothesize that a similar transcription factor may exist

for suberin deposition offering another potential epigenetic target that would induce downstream phenotypic change.

# 5 CONCLUSIONS & FUTURE DIRECTIONS

In this work I have successfully reduced expression of *CYP86A37* and *CYP86A38* using a *Rhizobium rhizogenes*-mediated hairy root system and RNAi-based constructs. Through subsequent analysis of aliphatic suberin deposition in transformed hairy roots, I established a role for *CYP86A37* and *CYP86A38* in biosynthesis of aliphatic suberin in soybean. Reducing expression of *CYP86A37* and *CYP86A38* only significantly impacted the amount of a single aliphatic suberin monomer, 18-hydroxy-oleic acid, and not total aliphatic suberin deposition. During development, *CYP86A37* and *CYP86A38* expression was linked to total aliphatic suberin content as well as the abundance of specific monomer types, particularly long chain length ω-OHs. This evidence supports identification of *CYP86A37* and *CYP86A38* as genes that encode functional fatty acid ω-hydroxylases with a preference for long chain length fatty acids (esp. oleic acid) as substrate.

Future experiments will need to focus on many facets that contribute to suberin biosynthesis including not only the genetic foundation, but also environmental and epigenetic factors that play a role in the suberin phenotype (Figure 5.1). Combining an understanding of a diverse set of factors will help to ensure a more complete understanding of what contributes to the suberin phenotype and ensure adequate targets when developing cultivars with specific suberin phenotypes that best equip the plants to combat ensuing environmental challenges.

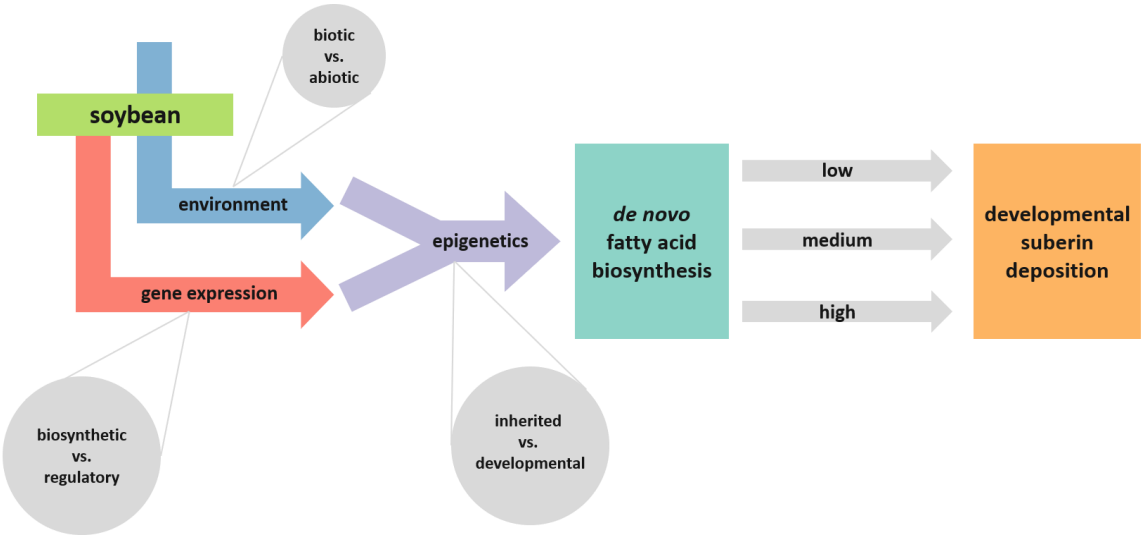


**Figure 5.1 Potential sources of factors impacting downstream developmental suberin deposition.** As suberin deposition is a quantitative trait, there are many genetic components that contribute this trait, including biosynthesis of precursors to both phenolic and aliphatic suberin monomers, the modification of precursor monomers, monomer transport, and the linkage into the polymeric structure. Then, regulation of these gene must also be considered as important elements that impact the suberin phenotype. In addition, it is also important to considering epigenetic factors that include both inherited impacts such as DNA methylation patterns as well as developmental changes in the epigenome like histone modifications. As these have the potential to alter expression of genetic factors, the epigenome ultimately will also contribute to the suberin phenotype. Finally, as the environment has the potential to alter the epigenome (including transgenerational impacts), it is important to include consideration of both abiotic and biotic environmental factors when thinking about what downstream suberin phenotype will result.

One critical step in understanding what contributes to the suberin phenotype in soybean would be further analysis of putative FAωHs. For example, instead of simply knocking down expression of *CYP86A37* and *CYP86A38*, knockout lines for these two genes could be generated. Total knockout could be achieved using CRISPR/Cas9 targeted genome modification approach as demonstrated by Jacobs et al. (2015), to knockout the genes both independently and in concert. It would also be helpful to determine whether differences in the expression of these two genes translates to downstream changes in protein abundance, which could be determined through protein extraction and western blotting.

It would also be prudent to confirm activity and substrate specificity of *CYP86A37* and *CYP86A38* through expression of recombinant protein and *in vitro* enzyme assays. Substrate specificity has been determined for several cytochrome P450 enzymes from Arabidopsis including AtCYP86A1, AtCYP86A2. AtCYP86A4, AtCYP86A7, and AtCYP73A5 (Benveniste et al. 1998; Duan et al. 2004; Rupasinghe et al. 2007). However, it is important to note that cytochrome P450 enzymes, and their assay are highly complex and therefore difficult to perform successfully. They require a eukaryotic system (i.e. yeast or transgenic virus infected flies) for proper protein folding, preparation of microsomes to preserve enzyme function for assays, the inclusion of an appropriate cofactor required for enzyme function in the assay (i.e. NADPH) and helper protein (cytochrome P450 reductase) (Benveniste et al. 1998; Duan et al. 2004; Rupasinghe et al. 2007). In addition to generating these additional observations for CYP86A37 and CYP86A38, it would also be prudent to explore similar experiments for the other putative soybean FAωHs predicted to be expressed in roots (e.g. CYP86B9, CYP94A17).

It will also be important to consider factors contributing to the suberin phenotype that are further upstream. For example, determining the developmental regulation of *CYP86A37* and *CYP86A38* expression. While biosynthesis of long chain length ω-OHs appears to play only a small role in the biosynthesis of aliphatic suberin, it is important to note that 18-hydroxy-oleic acid (a long chain length ω-OH) is the predominant monomeric unit of soybean aliphatic suberin and that regulation of *CYP86A37* and *CYP86A38* could also be shared by other suberin biosynthetic genes. One way to approach a deeper understanding of *CYP86A37* and *CYP86A38* regulation would be *in silico* analysis of the promoter regions of these two genes for motifs that correspond to specific types of transcription factors or hormonal regulators. In addition, considering that production of suberin specific monomers depends on availability of precursor molecules as substrates, a metabolomics approach could be used to determine whether differences in suberin phenotype can be linked to differences in substrate availability for biosynthesis of suberin monomers.

To better explore the persistence of the aliphatic suberin phenotype, it would be beneficial to look at suberin deposition across a wider range of cultivars with a larger sample size than what was used in this experiment. In considered both the data within this

experiment and Thomas et al. (2007) the variation of aliphatic suberin deposition within a cultivar can be quite high. By increasing sample size and range of cultivars, it would better establish the true range of aliphatic suberin deposition within each cultivar. This would assist in determining whether the total amount of aliphatic suberin deposited plays a role, or whether simply a given threshold level of suberin contribute the same amount to resistance regardless of cultivar. By also observing the suberin phenotype of each cultivar over several growing seasons, it would help to determine whether the phenotype of a single cultivar persists from year to year regardless of environmental differences in the field (e.g. presences vs. absence of a soil-borne pathogen like *P. sojae*). It would be important to monitor factors like plant mortality and changes in expression of key suberin biosynthesis genes, in additional to the suberin phenotype. If the suberin phenotype failed to persist across growing seasons and environment conditions, but it would prudent to also look for epigenetic differences like the changes in DNA methylation that is suggested to act as defense priming (Espinas et al. 2016). Changes in DNA methylation across the genome could be observed using methods like MeDIP-Seq (methylated DNA immunoprecipitation followed by sequencing).

In addition to looking more closely at the biosynthesis of suberin, it would also give further evidence for the role of suberin deposition in pathogen resistance if changes in expression of suberin biosynthesis genes could be linked to changes in the suberin phenotype. For example, it would be helpful to have a more comprehensive analysis of the suberin biosynthesis genes beyond *CYP86A37* and *CYP86A38*. The motivation for increasing our understanding of aliphatic suberin deposition in soybean was the previously described link between aliphatic suberin deposition and field level tolerance to *P. sojae*. While identification and preliminary functional characterization of *CYP86A37* and *CYP86A38* have increased our understanding of aliphatic suberin biosynthesis in soybean, the question of whether the expression of these two genes, and thus increased suberin deposition leads to increased resistance against soil-borne pathogens like *P. sojae* remains unanswered. In an attempt to answer this question, the three cultivars used in analysis of developmental suberin deposition and expression of *CYP86A37* and *CYP86A38* were inoculated with *P. sojae* as in (Thomas 2006) and chlorophyll fluorescence imaging (CFI) was used to monitor infection as in (Ivanov and Bernards 2015). However, the lack of

consistent response within a cultivar and irreproducibility of the results led to an inconclusive outcome. In addition, soybean has proven to be highly capable and maintaining homeostasis during light stress meaning small changes in plant health due to pathogen attack may go undetected using a CFI-based measure of plant health. Going forward, an attempt to directly link suberin biosynthesis genes to resistance a different plant-pathogen interaction could be used. For example, Arabidopsis offers a wealth of genetic tools with many mutants with different levels of suberin commercially available from the ABRC (i.e. *horst*, SALK_107454; *ralph*, SALK_130265; *esb1-1*, CS2106042; *myb36*, CS69049) with the same genetic background (*Col-1*, CS3176). In addition to these mutants, further suberin phenotypes could be obtained using complementation lines previously generated in the Bernards lab (Bjelica et al. 2016), where the *horst* mutant has been complemented with the potato ortholog. These plants could then be challenged with a pathogen like *Phytophthora parasitica* (Wang et al. 2011) and plant health be monitored using CFI.

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# Appendices

**Appendix A Overview of physiological impact and response to abiotic stress.** Includes information for the three most agronomically relevant abiotic stresses; drought, salinity, and extreme temperature.

| Reference | Stress | Physiological Impact | Response |
|---|---|---|---|
| Bita and Gerats (2011) | heat | - increased ROS production<br>- leaf scorching/senescence<br>- growth inhibition | - increased production of HSP, osmolytes, antioxidants<br>- shift in membrane lipids<br>- production of photoprotective pigments |
| Fang and Xiong (2015) | drought | - loss of turgor pressure<br>- water deficit | - stomatal closure<br>- increased water uptake (deeper roots)<br>- altered phenology<br>- altered internal osmolality<br>- production of antioxidants and ROS scavengers |
| Farooq et al. (2009) | drought | - loss of turgor pressure<br>- water deficit | - stomatal closure<br>- increased root depth<br>- altered leaf cuticle<br>- altered leaf surface – hairy leaves<br>- production of antioxidants<br>- altered internal osmolality<br>- altered expression of aquaporins and stress response proteins |
| Parihar et al. (2015) | salinity | - water deficit<br>- impaired nutrient availability | - altered expression of transporters<br>- production of osmoprotectants and osmolytes |
| Hatfield and Prueger (2015) | heat | - dehydration<br>- water deficit | - altered phenology |
| Heschel et al. (2017) | drought | - water deficit | - stomatal closure<br>- altered phenology |

| | | | |
|---|---|---|---|
| Madhulika et al. (2015) | salinity | - water deficit<br>- disruption of nutrient balance<br>- $Na^+$ toxicity<br>- increased ROS production | - production of osmoprotectants<br>o regulate osmotic adjustment<br>o deceased damage from ROS<br>o prevent membrane disruption<br>o stabilize enzymes and proteins |
| | drought | - water deficit<br>- disruption of nutrient balance<br>- increased membrane permeability<br>- denaturation of enzymes<br>- increased ROS production | |
| Mathur et al. (2014) | heat | - decreased growth<br>- decreased synthesis of photosynthetic apparatus | - production of stress response proteins – HSPs |
| Mostofa et al. (2015) | salinity | - increased ROS production<br>- water deficit | - production of osmoprotectants |
| Ohama et al. (2017) | heat | - increased ROS production<br>- protein denaturation | - production of HSPs (chaperones)<br>- synthesis of ROS scavengers |
| Parihor et al. (2015) | salinity | - interrupt membranes<br>- disrupts nutrient balance<br>- impairs ROS detoxification | - ion management<br>- production of osmoprotectants and antioxidants |
| Tochette et al. (2009) | salinity | - | - decrease stomatal conductance<br>- osmotic adjustment<br>- increased tissue rigidity |
| Tuteja (2009) | salinity, drought, cold | - denaturation of proteins<br>- increased ROS production<br>- $Na^+$ toxicity<br>- loss of membrane integrity<br>- chlorosis<br>- loss of turgor pressure<br>- water deficit | - decreased SA production – shed older leaves to decrease transpiration<br>- production of chaperone proteins and ROS scavengers |

**Appendix B Accession numbers and gene locus identifiers for the putative FAωHs identified in soybean.**

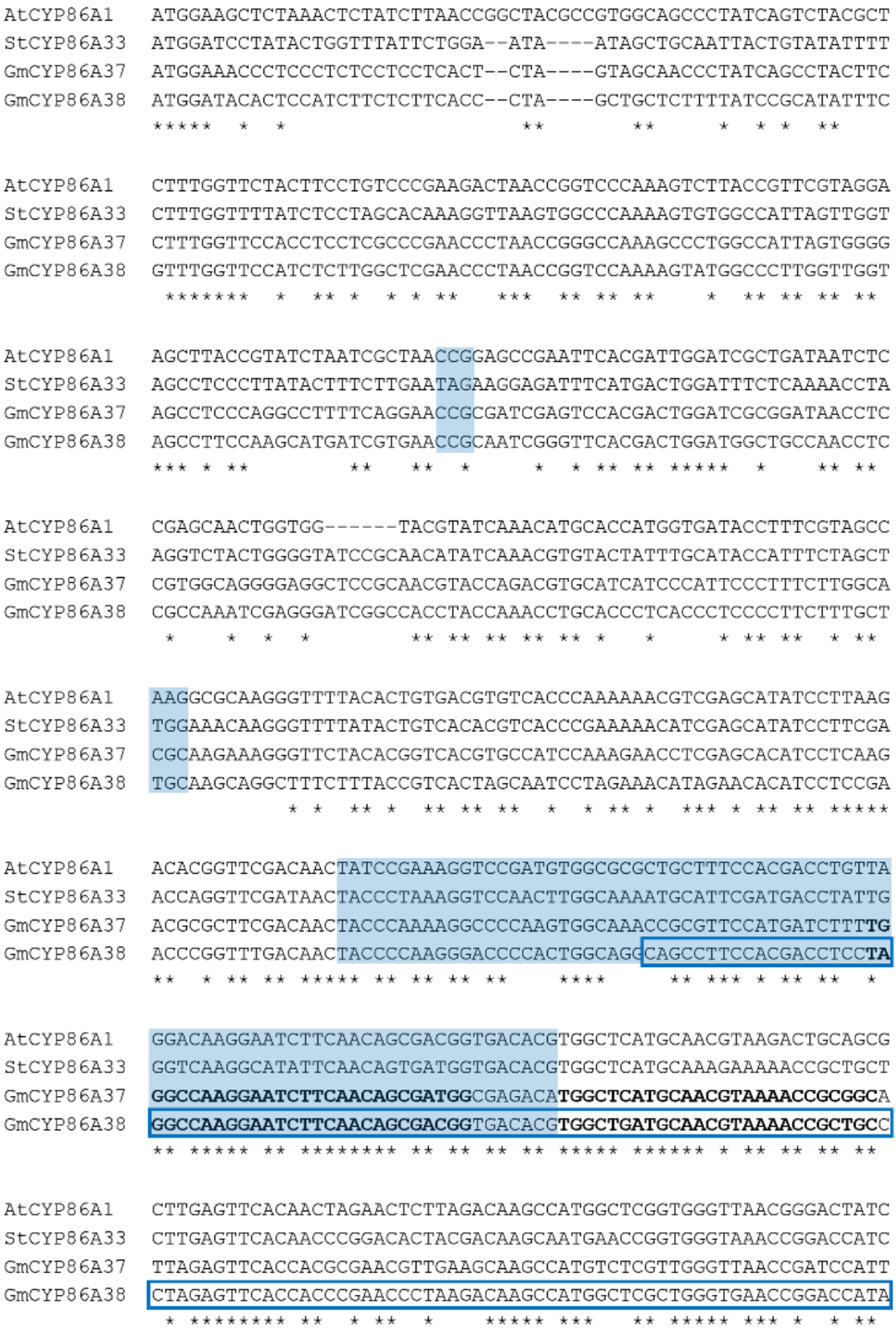
| Gene Name | NCBI accession number | Gene Locus (phytozome.net) | Gene Locus (soybase.org) |
|---|---|---|---|
| CYP86A37 | XP_003544876.2 | Glyma.14G192500.1 | Glyma14g37130 |
| CYP86A38 | XP_003538284.1 | Glyma.11G175900.1 | Glyma11g26500 |
| CYP86A65 | KRH41201.1 | Glyma.05G208900.1 | Glyma05g37700 |
| CYP86A24 | ABC68403.1 | Glyma.08G015600.1 | Glyma08g01890 |
| CYP86A67 | XP_006583288.1 | Glyma.07G069500.1 | Glyma07g07560 |
| CYP86A66 | XP_003521880.1 | Glyma.03G008100.1 | Glyma03g01050 |
| CYP86B9 | XP_003538960.2 | Glyma.11G100100.1 | Glyma11g10640 |
| CYP86B10 | XP_003533692.1 | Glyma.09G282700.1 | Glyma09g41940 |
| CYP86B11 | XP_003556602.2 | Glyma.20G002700.1 | Glyma20g00490 |
| CYP94A20 | XP_003542412.1 | Glyma.13G120500.1 | Glyma13g18110 |
| CYP94A19 | XP_014629305.1 | Glyma.03G160300.1 | Glyma03g31700 |
| CYP94A17 | XP_003520587.1 | Glyma.03G160100.1 | Glyma03g31680 |
| CYP94A21 | XP_003553484.1 | Glyma.19G162100.1 | Glyma19g34480 |
| CYP94A18P | XP_014629304.1 | Glyma.03G160200.1 | Glyma03g31691 |

**Appendix C RNA sequencing data compiled from Soybase.org for putative soybean FAωH genes across several tissue types.** Higher numbers indicate more reads and therefore higher expression of the given transcript in that tissue. No data was available for *CYP94A18P* (*Glyma03g31691*).

| Gene Name | Tissue Type | | | | | |
|---|---|---|---|---|---|---|
| | Root | Nodule | Young Leaf | Flower | Pod (1 cm) | Seed 21DAF |
| *CYP86A24* | 0 | 0 | 462 | 293 | 754 | 35 |
| *CYP86A37* | 284 | 67 | 1 | 7 | 14 | 7 |
| *CYP86A38* | 152 | 916 | 0 | 2 | 2 | 0 |
| *CYP86A65* | 0 | 7 | 155 | 201 | 357 | 47 |
| *CYP86A66* | 14 | 2 | 60 | 37 | 48 | 19 |
| *CYP86A67* | 11 | 6 | 86 | 33 | 34 | 15 |
| *CYP86B9* | 125 | 11 | 0 | 2 | 0 | 0 |
| *CYP86B10* | 0 | 278 | 0 | 10 | 78 | 84 |
| *CYP86B11* | 0 | 14 | 1 | 5 | 7 | 6 |
| *CYP94A17* | 87 | 0 | 0 | 1 | 3 | 0 |
| *CYP94A19* | 48 | 0 | 0 | 0 | 0 | 0 |
| *CYP94A20* | 13 | 2 | 13 | 54 | 73 | 12 |
| *CYP94A21* | 41 | 21 | 15 | 16 | 6 | 7 |

**Appendix D Gene specific primers for putative CYP86As in soybean.** Product sizes for *CYP86A37* and *CYP86A38* differ for both cDNA and gDNA as they have introns. For these two genes, the number indicated in bracket corresponds to the size on gDNA. The remaining four genes do not have introns and therefore the product sizes are the same for both cDNA and gDNA.

| Gene | Product size (nt) | Primers (5' → 3') | Number of Cycles | $MgCl_2$ (mM) | Annealing Temperature (◦C) |
|---|---|---|---|---|---|
| *CYP86A24* | 1833 | AGTCGCTCTGCTCGTTCGCTC<br>CAGAGGAAATGATACAGCACCGT | 31 | 4.38 | 57 |
| *CYP86A37* | 1339<br>(2215) | CAAGATATATGTGCATGTCCAC<br>TAATTCTGCAGCACAATGACG | 28 | 4.00 | 55 |
| *CYP86A38* | 1327<br>(2182) | AGAGATCGGCCACCTACCAAA<br>GAACACGCGGAGTCCATGCT | 27 | 4.00 | 65 |
| *CYP86A65* | 1625 | GGTCATATCAGGCTCTAGCTC<br>GGTTTGAGATCACTTGGGTACACATT | 32 | 4.38 | 60 |
| *CYP86A66* | 2012 | TGGTGCAGCTGATGAGAG<br>CCTCCAATACCCTATGCTTC | 28 | 2.50 | 56 |
| *CYP86A67* | 2013 | GGAGTATGGTGAGGCATTGTC<br>CCATCTAGTGAACACCTGGTC | 32 | 3.13 | 62 |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StCYP86A33 ATGGATCCTATACTGGTTTATTCTGGA--ATA----ATAGCTGCAATTACTGTATATTTT
GmCYP86A37 ATGGAAACCCTCCCTCTCCTCCTCACT--CTA----GTAGCAACCCTATCAGCCTACTTC
GmCYP86A38 ATGGATACACTCCATCTTCTCTTCACC--CTA----GCTGCTCTTTTATCCGCATATTTC
AtCYP86A1 CTTTGGTTCTACTTCCTGTCCCGAAGACTAACCGGTCCCAAAGTCTTACCGTTCGTAGGA
StCYP86A33 CTTTGGTTTTATCTCCTAGCACAAAGGTTAAGTGGCCCAAAAGTGTGGCCATTAGTTGGT
GmCYP86A37 CTTTGGTTCCACCTCCTCGCCCGAACCCTAACCGGGCCAAAGCCCTGGCCATTAGTGGGG
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StCYP86A33 AGCCTCCCTTATACTTTCTTGAATAGAAGGAGATTTCATGACTGGATTTCTCAAAACCTA
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GmCYP86A38 AGCCTTCCAAGCATGATCGTGAACCGCAATCGGGTTCACGACTGGATGGCTGCCAACCTC
AtCYP86A1 CGAGCAACTGGTGG------TACGTATCAAACATGCACCATGGTGATACCTTTCGTAGCC
StCYP86A33 AGGTCTACTGGGGTATCCGCAACATATCAAACGTGTACTATTTGCATACCATTTCTAGCT
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StCYP86A33 TGGAAACAAGGGTTTTATACTGTCACACGTCACCCGAAAAACATCGAGCATATCCTTCGA
GmCYP86A37 CGCAAGAAAGGGTTCTACACGGTCACGTGCCATCCAAAGAACCTCGAGCACATCCTCAAG
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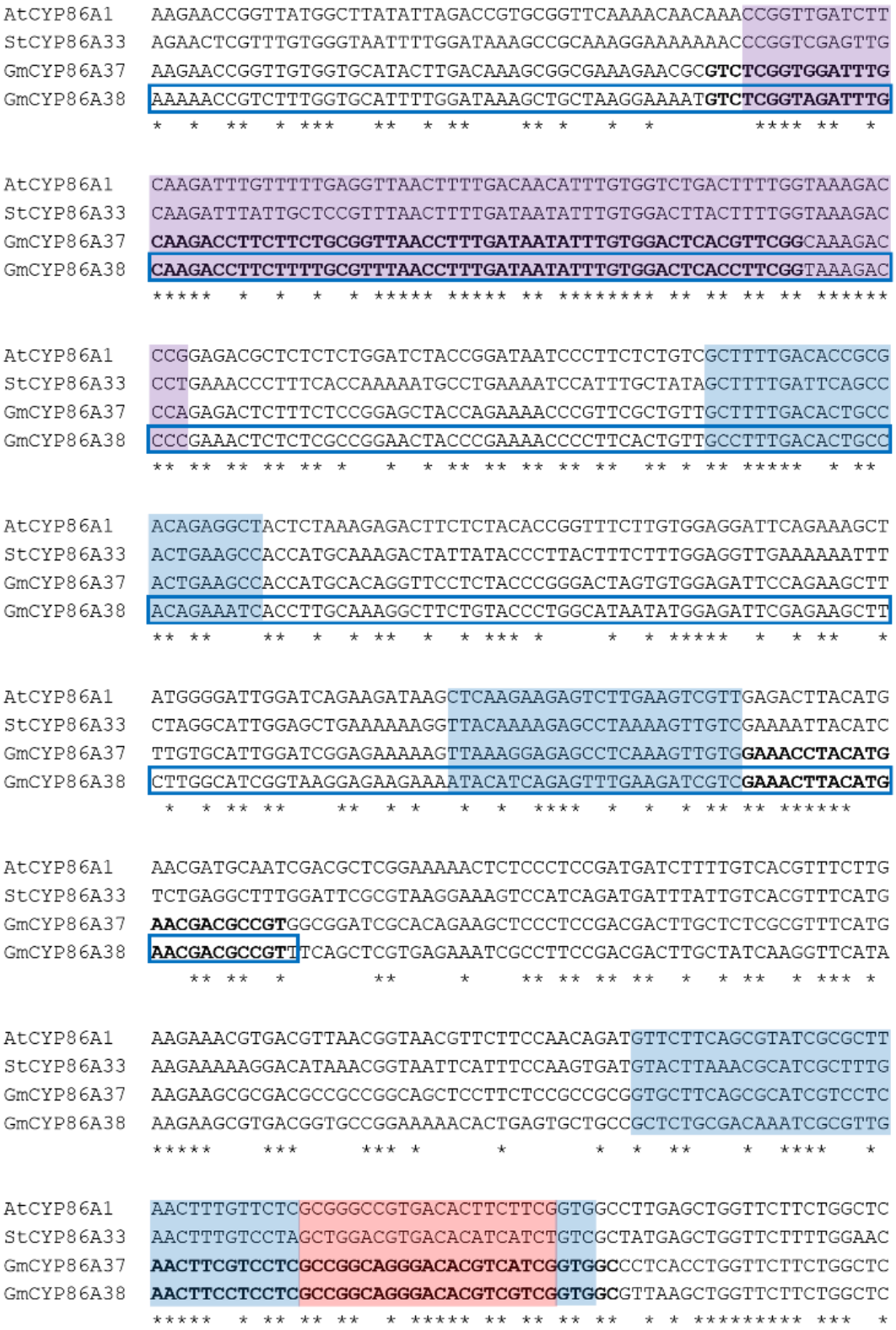
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GmCYP86A37 AAGAACCGGTTGTGGTGCATACTTGACAAAGCGGCGAAAGAACGCGTCTCGGTGGATTTG
GmCYP86A38 AAAAACCGTCTTTGGTGCATTTTGGATAAAGCTGCTAAGGAAAATGTCTCGGTAGATTTG
AtCYP86A1 CAAGATTTGTTTTTGAGGTTAACTTTTGACAACATTTGTGGTCTGACTTTTGGTAAAGAC
StCYP86A33 CAAGATTTATTGCTCCGTTTAACTTTTGATAATATTTGTGGACTTACTTTTGGTAAAGAC
GmCYP86A37 CAAGACCTTCTTCTGCGGTTAACCTTTGATAATATTTGTGGACTCACGTTCGGCAAAGAC
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AtCYP86A1 CCGGAGACGCTCTCTCTGGATCTACCGGATAATCCCTTCTCTGTCGCTTTTGACACCGCG
StCYP86A33 CCTGAAACCCTTTCACCAAAAATGCCTGAAAATCCATTTGCTATAGCTTTTGATTCAGCC
GmCYP86A37 CCAGAGACTCTTTCTCCGGAGCTACCAGAAAACCCGTTCGCTGTTGCTTTTGACACTGCC
GmCYP86A38 CCCGAAACTCTCTCGCCGGAACTACCCGAAAACCCCTTCACTGTTGCCTTTGACACTGCC
AtCYP86A1 ACAGAGGCTACTCTAAAGAGACTTCTCTACACCGGTTTCTTGTGGAGGATTCAGAAAGCT
StCYP86A33 ACTGAAGCCACCATGCAAAGACTATTATACCCTTACTTTCTTTGGAGGTTGAAAAAATTT
GmCYP86A37 ACTGAAGCCACCATGCACAGGTTCCTCTACCCGGGACTAGTGTGGAGATTCCAGAAGCTT
GmCYP86A38 ACAGAAATCACCTTGCAAAGGCTTCTGTACCCTGGCATAATATGGAGATTCGAGAAGCTT
AtCYP86A1 ATGGGGATTGGATCAGAAGATAAGCTCAAGAAGAGTCTTGAAGTCGTTGAGACTTACATG
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GmCYP86A37 TTGTGCATTGGATCGGAGAAAAAGTTAAAGGAGAGCCTCAAAGTTGTGGAAACCTACATG
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GmCYP86A37 AACTTCGTCCTCGCCGGCAGGGACACGTCATCGGTGGCCCTCACCTGGTTCTTCTGGCTC
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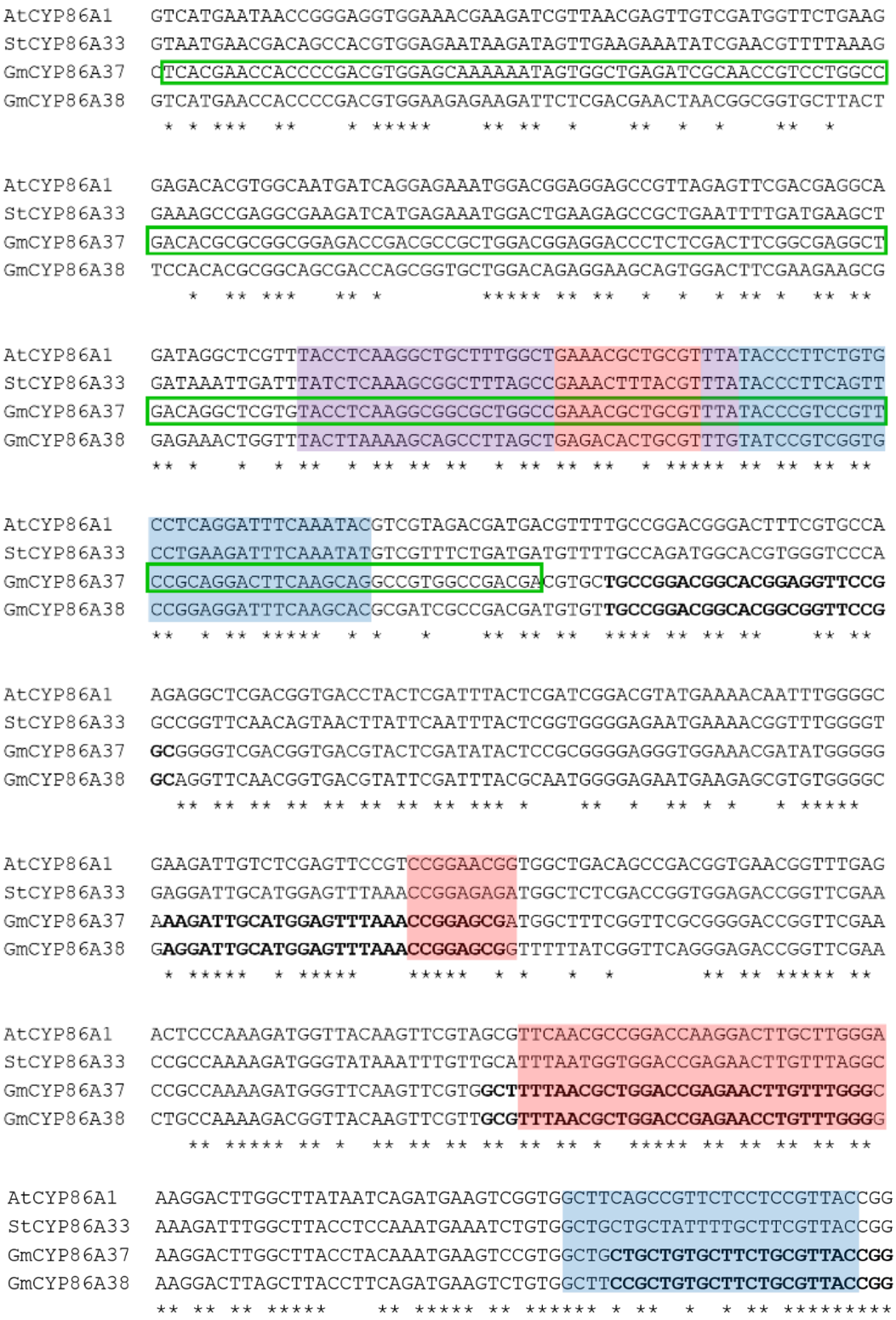
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GmCYP86A38 GTCATGAACCACCCCGACGTGGAAGAGAAGATTCTCGACGAACTAACGGCGGTGCTTACT
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StCYP86A33 GAGGATTGCATGGAGTTTAAACCGGAGAGATGGCTCTCGACCGGTGGAGACCGGTTCGAA
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GmCYP86A38 GAGGATTGCATGGAGTTTAAACCGGAGCGGTTTTTATCGGTTCAGGGAGACCGGTTCGAA
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StCYP86A33 CCGCCAAAAGATGGGTATAAATTTGTTGCATTTAATGGTGGACCGAGAACTTGTTTAGGC
GmCYP86A37 CCGCCAAAAGATGGGTTCAAGTTCGTGGCTTTTAACGCTGGACCGAGAACTTGTTTGGGC
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StCYP86A33 AAAGATTTGGCTTACCTCCAAATGAAATCTGTGGCTGCTGCTATTTTGCTTCGTTACCGG
GmCYP86A37 AAGGACTTGGCTTACCTACAAATGAAGTCCGTGGCTGCTGCTGTGCTTCTGCGTTACCGG
GmCYP86A38 AAGGACTTAGCTTACCTTCAGATGAAGTCTGTGGCTTCCGCTGTGCTTCTGCGTTACCGG

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AtCYP86A1   GTTTTTCCGGTTCCCGGTCACCGCGTTGAGCAAAAGATGTCGCTGACGCTTTTCATGAAG
StCYP86A33  CTATTACCGGTTCCCGGTCATAAAGTTGAACAGAAAATGTCGTTGACTTTGTTCATGAAA
GmCYP86A37  CTATCGCTGGTTCCCGGTCACCGGGTGGAACAGAAGATGTCTCTCACTTTGTTCATGAAG
GmCYP86A38  CTGTCGCCGGTTCCCGGTCACCGGGTGCAGCAGAAGATGTCGCTCACTCTCTTCATGAAG
             * *  * ************    **  * ** ** *****  * **  * ********

AtCYP86A1   AACGGTTTACGTGTTTATTTGCAACCTCGTGGTGAGGTGCTTGCATGATTCACATTAAGG
StCYP86A33  AATGGACTTAAAGTTTACTTGAATCCACGTGAACTTGATCTTGCAGCTCCAAAGATTGCT
GmCYP86A37  AATGGGCTCAGGGTGTTCTTGCATCCACGTAAGCTAGAAAGTGGGCCCGGGGTTGCCACC
GmCYP86A38  CATGGGCTCCGCGTGTTCTTGCAGACACGGTGA--------------------------
             * **  *    ** *  *** *  * **

AtCYP86A1   GTATTATCGTAAATATGTTTGTCATTTGAAGTTTAAATTGTTTTAAGAACAACCTACTTT
StCYP86A33  ATGTCTGCATGAAGAAAGGGTAAAATGGGAATTAAAATGGTATAATGGAATTATTGGTAA
GmCYP86A37  TCACCTTAA-----------------------------------------------------
GmCYP86A38  -----------------------------------------------------------

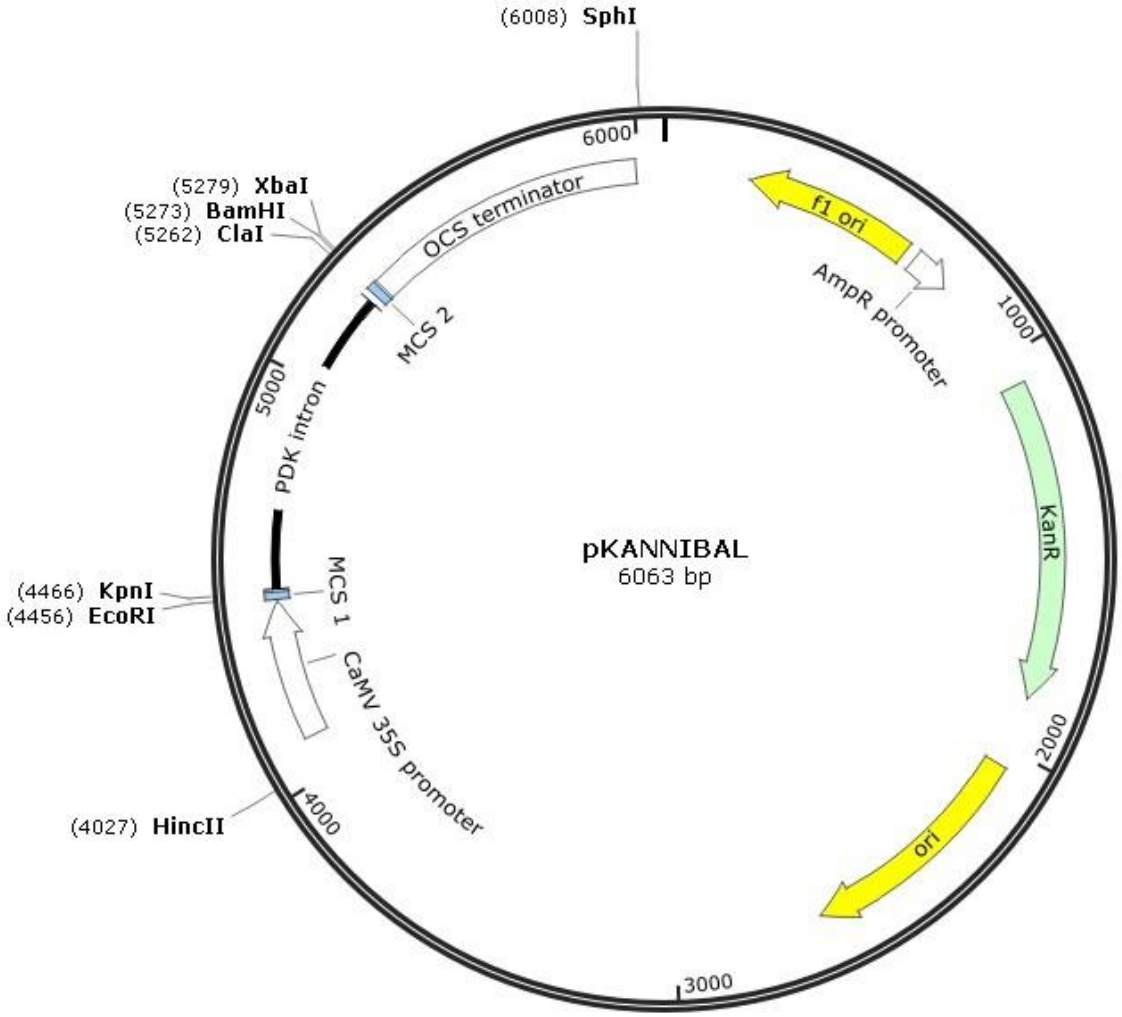
AtCYP86A1   ATTCTATGTTTTCTCCACCTACAAAATACTTATTATGATATATATGCACTTTTTGTGCCT
StCYP86A33  TTGTAATTGTATTTGCTTTGTTGTAATTTTCAATGTG----------------------
GmCYP86A37  -----------------------------------------------------------
GmCYP86A38  -----------------------------------------------------------

AtCYP86A1   TTGTTTGTTTGAGTTTGTTCAACTCTTTTTGAATAAAAGTTTATTTGGTTTAACTTGATG
StCYP86A33  -----------------------------------------------------------
GmCYP86A37  -----------------------------------------------------------
GmCYP86A38  -----------------------------------------------------------

AtCYP86A1   AATACAAAAACAAAAT
StCYP86A33  ----------------
GmCYP86A37  ----------------
GmCYP86A38  ----------------
```

**Legend**
- substrate recognition sites
- conserved across FAωHs
- conserved across all plant P450s
- sequence selection for pHR5only
- sequence selection for pHR5&6

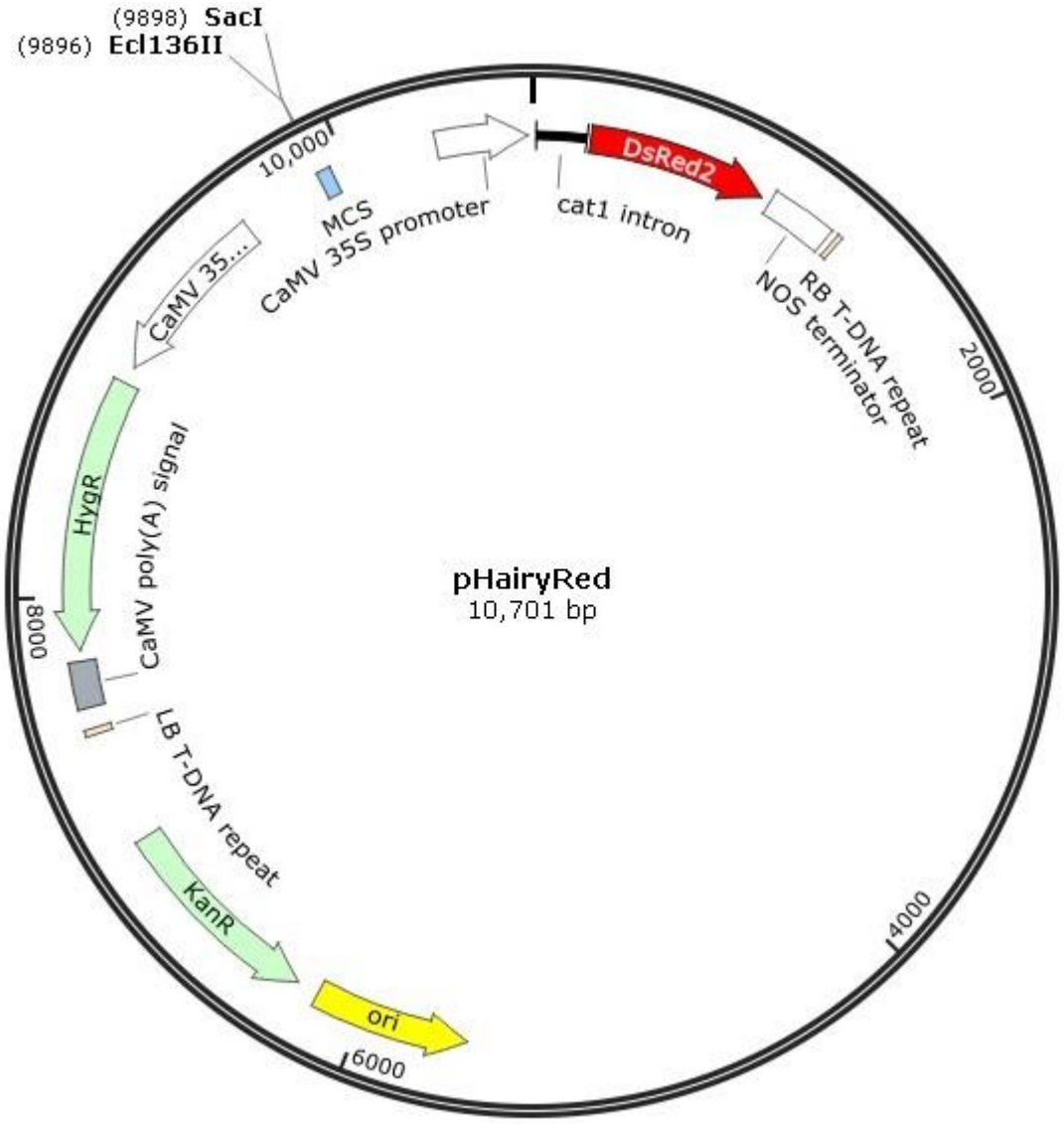
**Appendix E Nucleotide sequence alignment of exonic region of AtCYP86A, StCYP86A33, GmCYP86A37, and GmCYP86A38 (DNAMAN).** Dashes indicate gaps introduced by the alignment and bolded characters indicated ≥ 21 nt fragments with near perfect sequence identity between GmCYP86A87 and GmCYP86A38. While efforts were made to avoid areas of high homology, some overlap was required to have long enough fragment lengths (200-600 nt) to generate constructs. It is also important to note that when overlap occurs, it is generally with regions that are limited to the FAωH superfamily, or 86A subfamily, and not regions that are conserved across all P450 enzymes.



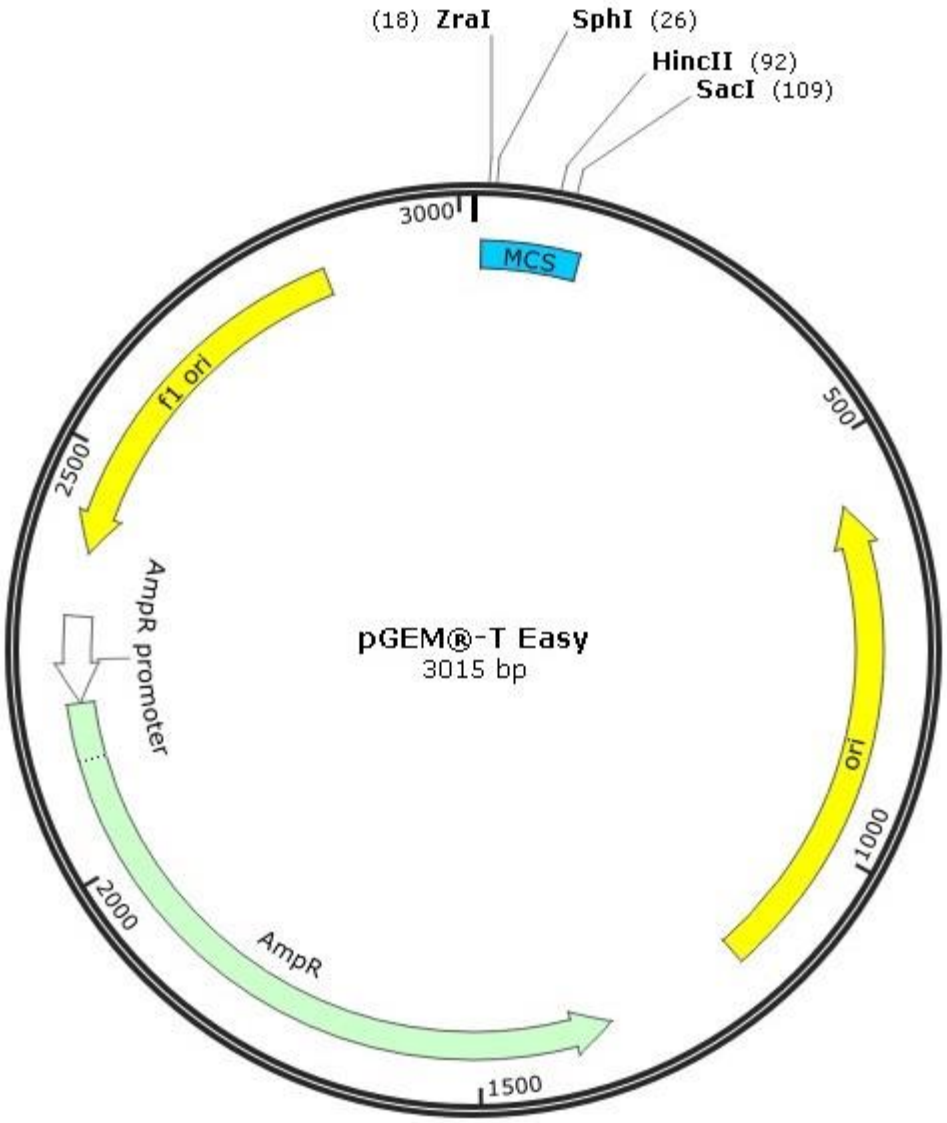

**Appendix F Vector map for cloning vector pKANNIBAL.** This vector was used to clone the inverted repeat to be used in the RNAi knockdown of *CYP86A37* and *CYP86A38*. Only the restriction enzyme sites that were used in this thesis have been included on the map. (Wesley et al. 2001)

**Appendix G Primers for the amplification of gene fragments used in the generation of RNAi knockdown constructs.** Cloning of an inverted repeat requires sequential cloning where the fragment is cloned first in one orientation (underlined RE cut sites) and then in the second orientation (italicized RE cut sites). Primer nucleotides in bold are complementary to the template used for amplification.

| Target(s) | Template | Primers (5' → 3') | Position (nt) | Amplicon length (nt) | REs cut #1 | REs cut #2 |
|---|---|---|---|---|---|---|
| *CYP86A37* | *CYP86A37* | ATT*GGATCC*GGTA**CC**TCACGAAC<br>TCA*TCTAGA*GAAT**TC**GTCGGCCAC | 953-1166 | 249 | EcoRI<br>KpnI | BamHI<br>XbaI |
| *CYP86A37*<br>*CYP86A38* | *CYP86A38* | TTA*GGTACC*ATCGAT**CAGCCTTCCA**<br>CGATT*GAATTC*TCTAGA**AACGGCGTC** | 344-795 | 483 | ClaI<br>XbaI | EcoRI<br>KpnI |




**Appendix H Vector map for cloning vector pHairyRed.** This vector was as the final destination vector for transformation of soybean to generate hairy roots. Only the restriction enzyme sites that were used in this thesis have been included on the map. (Lin et al. 2011)




**Appendix I Vector map for cloning vector pGEM-T Easy.** This vector was used as an intermediate to transfer the inverted repeats generated using pKANNIBAL to the destination vector to be used in hairy root transformation, pHairyRed. Only the restriction enzyme sites that were used in this thesis have been included on the map (Promega).

**Appendix J Gene specific primers for qRT-PCR.** The two genes of interest are *CYP86A37* and *CYP86A38* and the reference genes are *cons6* and *cons7* (Libault et al., 2008). Expected product size is based on amplification using cDNA as a template.

| Gene | Product size (nt) | Primers (5' → 3') | Primer efficiency |
|---|---|---|---|
| *cons6* | 93 | AGATAGGGAAATGGTGCAGGT<br>CTAATGGCAATTGCAGCTCTC | 114.2 |
| *cons7* | 114 | ATGAATGACGGTTCCCATGTA<br>GGCATTAAGGCAGCTCACTCT | 101.3 |
| *CYP86A37* | 169 | GTGCCATCCAAAGAACCTC<br>CGTGGTGAACTCTAATGCC | 92.2 |
| *CYP86A38* | 107 | GGCATCGGTAAGGAGAAG<br>GATAGCAAGTCGTCGGAAG | 90.3 |

**Appendix K Gene specific primers for qRT-PCR.** The two genes of interest are *CYP86A37* and *CYP86A38* and the reference genes are *ACT-II*, *cons4*, *cons6*, and *cons7* (Libault et al., 2008). Expected product size is based on amplification using cDNA as a template.

| Gene | Product size (nt) | Primers (5' → 3') | Primer efficiency |
|---|---|---|---|
| *ACT-II* | 126 | ATCTTGACTGAGCGTGGTTATTCC<br>GCTGGTCCTGGCTGTCTCC | 121.9 |
| *Cons 4* | 106 | GATCAGCAATTATGCACAACG<br>CCGCCACCATTCAGATTATGT | 108.5 |
| *Cons6* | 93 | AGATAGGGAAATGGTGCAGGT<br>CTAATGGCAATTGCAGCTCTC | 130.0 |
| *Cons7* | 114 | ATGAATGACGGTTCCCATGTA<br>GGCATTAAGGCAGCTCACTCT | 109.7 |
| *CYP86A37* | 169 | GTGCCATCCAAAGAACCTC<br>CGTGGTGAACTCTAATGCC | 104.2 |
| *CYP86A38* | 107 | GGCATCGGTAAGGAGAAG<br>GATAGCAAGTCGTCGGAAG | 114.7 |

# Curriculum Vitae

**Name:** Trish Tully

**Post-secondary Education and Degrees:**

The University of Western Ontario
London, ON, Canada
2009-2013 B.Sc. Honours Specialization Genetics

The University of Western Ontario
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2013-Present Ph.D. Biology

**Honours and Awards:**

Ontario Graduate Scholarship (OGS)
2014-2015, 2017-2018

Department of Biology Travel Award
For: The International Symposium on Plant Apoplastic Diffusion Barriers (PADiBa)
2017

4th Best Poster Award
The Southwestern Ontario Regional Association of the Canadian Pathological Society (SORA-CPS)
2016

2nd Best Poster Award
SORA-CPS
2015

**Related Work Experience**

Teaching Assistant
The University of Western Ontario
2009-2013

Research Assistant
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**Conference Presentations:**

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*Oral Presentations*

TLA Tully*, P Kaushik, J O'Connor, & MA Bernards (2018) Characterization of CYP86A genes and their role aliphatic suberin deposition in soybean roots. The Eastern Regional Meeting of the Canadian Society of Plant Biologists (CSPB). London, ON, Canada.

TLA Tully*, P Sharma, J Koteles, MA Bernards (2017) Linking FAωH gene expression to suberin deposition in soybean: a step towards future. The International Symposium on Plant Apoplastic Diffusion Barriers (PADIBA17). Oeiras, Portugal.

TLA Tully*, A, Bjelica, & MA Bernards (2015) Soybean root suberin and resistance against the pathogen *Phytophthora sojae*. The 6th Annual Biology Graduates Research Forum (BGRF). London, ON, Canada.

MA Bernards*, TLA Tully, & A Bjelica (2015) Soybean root suberin and resistance against the pathogen *Phytophthora sojae* . The 7th European Symposium on Plant Lipids. Harpenden, UK.

TLA Tully*, A Bjelica, & MA Bernards. (2014) Soybean root suberin and resistance against the pathogen *Phytophthora sojae*. The 21st International Symposium on Plant Lipids (ISPL). Guelph, ON, Canada.

TLA Tully*, J Koteles, PK Sharma, & MA Bernards (2013) Expression of fatty acid ω-hydroxylase genes in soybean (*Glycine max*). The 4th Annual Biology Graduates Research Forum. London, ON, Canada.

MA Bernards*, CA Peterson, RH Thomas, K Ranathunge, S Shao, M Gijzen, A Fang, F Ma, CJ Meyer, & TLA Tully (2013) Diverse functions of diffusion barriers in roots and seeds. The International Symposium on Plant Apoplastic Diffusion Barriers (PADIBA13). Lausanne, Switzerland.

TLA Tully, J Koteles, PK Sharma, & MA Bernards* (2013) Hairy roots as a model to investigate the role of suberin in soybean resistance to *Phytophthora sojae*. The 52nd Annual Meeting of the Phytochemical Society of North America (PSNA). Corvallis, USA.

*Poster Presentations*

TLA Tully*, A Bjelica, MA Bernards (2016) The role of suberin in resistance against the pathogen *Phytophthora sojae*. The Southwestern Ontario Regional Association of the Canadian Pathological Society. Vineland, ON, Canada.

TLA Tully*, A Bjelica, MA Bernards (2016) Root suberization in soybean and its role in resistance against the pathogen *Phytophthora sojae*. The Symposium on Sustainable Agriculture. London, ON, Canada.

TLA Tully*, A, Bjelica, & MA Bernards (2015) Soybean root suberin and resistance against the pathogen *Phytophthora sojae*. Southwestern Ontario Regional Association of the Canadian Phytopathological Society (SORA-CPS) Annual Meeting. London, ON, Canada.

TLA Tully* and MA Bernards (2014) Soybean root suberin and resistance to Phytophthora sojae. Sustainability and Environment Research Showcase. London, ON, Canada.