Investigating the role of *BLADE-ON-PETIOLE* genes and hydrophobic cell-wall polymer suberin in *Arabidopsis thaliana* defense against bacterial and fungal pathogens

By

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ABSTRACT

In this thesis, I use the model plant species, *Arabidopsis thaliana* (Arabidopsis), to examine disease resistance associated with *BLADE-ON-PETIOLE (BOP) 1* and *2* genes and suberin polymer. Plants are the target of a broad spectrum of pathogens, including fungi and bacteria. To detect and actively fight these pathogens, plants have innate and induced immune systems as well as pre-formed barriers. Firstly, I examined the role of the *BOP1/2* genes of Arabidopsis in plant defense against bacterial and fungal pathogens. *BOP1/2* genes are known to control plant development and belong to an evolutionarily conserved subclade of BTB-ankyrin transcription co-factors in plants. A paralog of BOP1/2, NPR1 is a positive regulator of the plant defense response known as systemic acquired resistance (SAR). Using a direct pathogen test approach to examine the resistance and susceptibility of *BOP1/2* mutants to *Pseudomonas syringae* and *Botrytis cinerea,* I found that BOP1/2 have a role in defense against pathogens, and specifically in innate immunity. Secondly, I examined the role of the hydrophobic cell-wall polymer suberin in protecting Arabidopsis against pathogenic bacteria and fungi. Suberin forms a complex extracellular hydrophobic lipid-based barrier that is deposited in various tissues in terrestrial plants. Suberin serves as a protective barrier against abiotic and biotic environmental stresses and plays important roles in controlling water and ion movement. Mutants of Arabidopsis defective in root suberin, as well as mutants ectopically overexpressing suberin were tested in relation to resistance or susceptibility to both fungal and bacterial pathogens. Root suberin mutants differed from wild type control plants in susceptibility to the fungal pathogen *Fusarium avenaceum.* Strikingly, I also found that the presence of suberin in the leaves of Arabidopsis promotes *Botrytis cinerea* infection spread as well as water loss in the leaves of the plants.

PREFACE

This thesis is about the role of organ boundary genes (previously known for developmental functions) and suberin – (a complex extracellular hydrophobic lipid-based cell wall deposition) in plant defense, using the plant model species *Arabidopsis thaliana.*

The research work of the thesis was independently carried out by me in the Department of Biology, Carleton University under the co-supervision of Dr. Myron Smith and Dr. Shelley Hepworth who conceived and designed this project, and they also edited my thesis.

I would also like to thank Chris Bergin of the Hepworth lab for his preliminary analysis of the transcriptome of *BOP1* overexpressing plants using Gene Ontology to find evidence of a role for BOPs in plant defense. These data served as a basis for my project.

Part of the work described in this thesis has been submitted for publication.

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LIST OF ABBREVIATIONS

ABA – Abscisic acid

ABC - ATP-binding-cassette

ABRC - Arabidopsis Biological Resource Centre

AHC - Alkyl hydroxycinnamate

ANOVA - Analysis of variance

ASFT - Aliphatic suberin feruloyl transferase

BAK1 – BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1

BIK1- BOTRYTIS-INDUCED KINASE 1

BOP - BLADE-ON-PETIOLE

bZIP – basic leucine zipper

CB – Casparian band

CBP- CALMODULIN-BINDING PROTEIN

CD - cutin deficient

CDPK – CALCIUM DEPENDENT PROTEIN KINASE

Col – Columbia

CYP - Cytochrome P450 monooxygenase

DAMP – Damage associated molecular pattern

DCA - Dicarboxylic acid

ER - Endoplasmic reticulum

ET - ethylene

FA - Fatty acid

FACT - Fatty alcohol caffeoyl-CoA transferase

FAE - Fatty acid elongation

FAR - Fatty acyl reductase

FLS – FLAGELLIN-SENSITIVE

FRK1 – FLG22-INDUCED RECEPTOR-LIKE KINASE

G3P - Glycerol-3-phosphate

GA – gibberellin

GO – Gene Ontology

GLS- GLUCAN SYNTHASE-LIKE

GPAT - Glycerol 3-phosphate acyltransferase (GPAT)

JA – jasmonic acid

KCS - Ketoacyl-CoA synthase

LOB – Lateral organ boundary

MAMP – microbe associated molecular pattern

MAPK – MITOGEN ACTIVATED PROTEIN KINASE

NAC - NAM/ATAF1/2/CUC2

NHL10 – NDR1/HIN1-LIKE 10

NPR1 – NON-EXPRESSER OF PATHOGENESIS RELATED GENE 1

OH-FA - Omega hydroxy fatty acid

PAMP – pathogen associated molecular pattern

PTI – PAMPs triggered immunity

PR – PATHOGENESIS-RELATED

RBOHD – RESPIRATORY BURST OXIDASE HOMOLOGUE D

ROS – reactive oxygen species

RWC - Relative water content

SA – salicylic acid

SAM - shoot apical meristem

SAR – systemic acquired resistance

SARD- SAR DEFICIENT

SDS – sodium dodecyl sulphate

SPAD – Suberin poly-aliphatic domain

SPPD - Suberin poly-phenolic domain

TEM - Transmission electron microscopy

TGA – TGACG

UDP – uridine diphosphate

VLCFA - Very-long-chain fatty acid

WRKY – WRKY domain-containing

WT – wild-type

CHAPTER 1: Introduction

1.1 Thesis overview

Arabidopsis thaliana (Arabidopsis) is a model plant species of the Brassicaceae family. Other members of this family include important agricultural crops such as mustard and canola. Crop plants often experience biotic and abiotic stresses, which can cause great loss in plant productivity and threaten our food security (Cerda et al., 2017; Misra & Chaturvedi, 2015; Savary et al., 2006). Biotic stresses include attacks by herbivores such as insects and plant pathogenic microorganisms such as viruses, bacteria, fungi and oomycetes (Teixeira et al., 2019). Plant pathogens, in particular, can dramatically reduce biomass output of key staple food crops, and also lead to a reduction in the general output of agriculture (Van de Weyer et al., 2019). Researchers and breeders therefore endeavor to study and understand how plants defend themselves against pathogens, with an aim to improve crop productivity and food security.

To repel, detect, and actively fight pathogens, plants have tiered defense systems which include pre-formed barriers as well as innate and induced immune systems. A plant's defense against diseases begins with preformed barriers that prevent pathogen entrance and invasion. Among these natural barriers are the plant cell wall, the cuticle (an extracellular surface coating that is mainly made up of the polymer cutin and its associated waxes), and suberin with its associated waxes (Vishwanath et al., 2015). Further success of the plant depends on an ability to recognize an invading pathogen and mount a resistance that prevents the establishment of infection (Schenk et al., 2000). Plants largely depend on the use of both localized (innate) defense responses as well as systemic (induced) defense responses to ward off intruding pathogens. Defense responses have been well studied in the model organism Arabidopsis. Innate responses are based on cell surface receptors that

recognize conserved molecules associated with pathogens (Henry et al., 2013; Spoel & Dong, 2012). The systemic immunity, also known as systemic acquired resistance, is a broad-spectrum type of immunity that is controlled by the BTB-ankyrin transcription co-factor protein NONEXPRESSER OF PATHOGENESIS RELATED GENES1 (NPR1) (Spoel & Dong, 2012). Arabidopsis NPR1 belongs to a small family of proteins that play an essential role in the effectiveness of plant immune responses (Backer et al., 2019). Included in this family are BLADE-ON-PETIOLE 1 and 2 (BOP1/2). BOP1/2 are primarily known as boundary organ genes and they interact with the same transcription factors that NPR1 interacts with, but they function in plant growth and development (Hepworth et al., 2005; Khan et al., 2014).

My thesis is divided into two sections. In the first section, I explore the roles of the Arabidopsis boundary genes *BOP1/2* in both innate and acquired immunity against bacterial and fungal pathogens. In the second section, I investigated the potentials for hydrophobic suberin polymer deposition in Arabidopsis to serve as both physical and biochemical barriers against invasion by fungal and bacterial pathogens.

1.2 Arabidopsis - a model species for studies in plant biology

Arabidopsis thaliana L. is most commonly referred to as 'Arabidopsis' but is also known as mouse ear cress or thale cress. Arabidopsis is a model plant with extensive resources that has had a profound impact on the field of plant science. It is a small dicotyledonous plant belonging to the mustard family (Brassicaceae) and is closely related to vegetable crops such as cabbage, broccoli and radish, and to oilseed crops like canola. Arabidopsis has been used for research purposes since as early as 1900 (Provart et al., 2016) and is favored as a model in all areas of plant biology, including genetics, physiology,

biochemistry and development. Information obtained from Arabidopsis has been successfully applied to improve many crop species (Woodward & Bartel, 2018). Some of the qualities that make Arabidopsis a prominent model organism for research include its ease of growth under controlled conditions, small size of plants of about 30 cm high at maturity, and short generation time of approximately two months from germination to seed dispersal. Further, it has a relatively small genome size (approximately 115 Mb) which has been sequenced and annotated (Kaul et al., 2000) and this information is readily available through open access databases such as www.arabidopsis.org (The Arabidopsis Information Resource). The genome contains about 20,000 genes, which are organized into five chromosomes. As another advantage, Arabidopsis seed production is abundant via self-pollination or cross pollination (Provart et al., 2016). The induction of gene mutations in Arabidopsis is efficiently performed through the use of chemical mutagens, transformation with *Agrobacterium tumefaciens* to introduce T-DNA insertions, and the use of gene editing technologies such as CRISPR-Cas9 (Provart et al., 2016). The first systematic description of mutants was initiated in about 1945 (Saeidfirozeh et al., 2018), and Arabidopsis databases now consist of thousands of mutant and transgenic lines with characterized mutations. These lines are appropriate for diverse studies on plant growth and development, responses to environmental stimuli, responses to biotic and abiotic signals, and other areas of research.

Arabidopsis also provides an ideal genetic system with which to understand mechanisms involved in plant immunity and has been previously used as a model for studying plant – pathogen interactions (Krämer, 2015). Arabidopsis was the first plant used in the isolation of plant immune receptor genes, to show hormone-related resistance

pathways, and to develop the notion of basal defense. Research and improvement on other plant systems has proceeded from the pioneering work with Arabidopsis. As a result, important crop traits including resistance to biotic (pathogens and pest) and abiotic stresses such as drought and salinity have been improved, with overall improvements in crop production and yield (Ferrier et al., 2011; Provart et al., 2016).

In my study, Arabidopsis was employed to test the role of *BLADE-ON-PETIOLE* genes in plant immunity, as well to test possible suberin roles in pathogen resistance.

1.3 The plant immune system – general overview

Plants are frequently under threat of attack by pathogens in nature (Doehlemann & Hemetsberger, 2013), and have evolved defense strategies, including a variety of constitutive and inducible systems, to prevent colonization by invading pathogens (De Wit, 2007; Jones & Dangl, 2006). Plants generally do not use only one method to self-fortify against pathogens but have evolved multiple different layers of defense. Plant defense begins with preformed, constitutive defenses which include natural external barriers such as bark and waxy extracellular surface coatings like the cuticle (Grennan, 2006). The plant cell wall also has a protective function along with associated depositions of lignin or suberin. All of these barriers can interfere with and prevent the penetration of pathogens into the plant tissue. Constitutive defenses are also based on the action of chemicals (phytoanticipins), which possess antimicrobial, anti-insect, antifeedant or other inhibitory activities and are present in plants prior to infection (Lukas Schreiber, 2010; Shamrai, 2014). When a pathogen is able to overcome these pre-existing barriers, plants may then employ localized and systemic immune responses, to detect, ward off, and execute a

response that prevents further colonization and growth of the pathogen (Backer et al., 2019; Glazebrook, 2005; Grennan, 2006; Nurnberger et al., 2004).

1.4 Preformed natural external barriers (passive outer barriers)

The first line of defense in plants is an intact external physical barrier composed of the bark (stems) or a waxy cuticle (leaves), which protect plants against intruding microbes or mechanical damage by chewing insects (Figure 1-1). The bark is the outermost layer of a woody stem and is made up of layers of dead cells (Aronson et al., 2009). Bark tissues consists of various biopolymers including tannins, lignin, suberin, and polysaccharides. Vane et al. (2006) proposed that the suberin in bark acts as a barrier to microbial degradation by white-rot fungus *Lentinula edodes*. Leaves are covered with waxy cuticle which also helps to prevent the underlying cells from becoming infected by pathogens. The plant cuticle is a complex structure that is mainly composed of waxes and a polyester called cutin. Cutin is a mixture of very long-chain fatty acids and their derivatives embedded in antimicrobial substances (Kolattukudy, 1980; Serrano et al., 2014). Waxes are complex mixture of alcohols, hydrocarbon, aldehydes, ketones, esters, acids and are insoluble in water but soluble in a variety of organic solvents (Seigler & Seigler, 1998; Yeats et al., 2012). Waxes can be deposited within the cuticle or on the plant surface where they give a shiny appearance. These hydrophobic barriers serve to protect plants against water loss and various abiotic and biotic stresses (Bernard & Joubès, 2013).

1.4.1 Plant cell wall as a barrier against pathogen invasion

The cell wall provides the plant with structural support and rigidity but is also a preformed protective barrier that can limit pathogen access into plant cells. Potential plant pathogens must overcome the cell wall barrier to access the interior of cells and to colonize the internal plant tissues (Underwood, 2012). The plant cell wall is dynamic in nature and can control the outcome of plant-pathogen interactions (Bellincampi et al., 2014). The plant cell wall may be differentiated into primary and secondary cell wall layers, that differ in function and in composition. The primary cell walls are synthesized during growth and are typically thin, flexible and strong to allow for expansion during growth and also withstand the ductile forces arising from turgor pressure. As the cell develops, it may undergo expansion and structural reinforcements, which lead to the formation of the secondary cell wall. The secondary cell wall is more rigid and responsible for the plant's mechanical support, providing strength and rigidity in plant tissues that have stopped growing (Cosgrove & Jarvis, 2012; Hamant & Traas, 2010). The primary cell wall consists mainly of carbohydrate-based polymers such as cellulose, hemicellulose and pectins, along with structural proteins which determine subtle properties of the wall. For example, the flexible rod-like extensin proteins are the most studied cell wall protein. These proteins are essential for cell wall assembly and growth by cell extension and expansion (Grennan, 2006; Schnabelrauch et al., 1996; Srivastava, 2002). The secondary cell wall is primarily made up of cellulose and hemicelluloses like xylans as well as lignin (Cosgrove, 2001; Cosgrove, 2005; Miedes et al., 2014).

The plant cell wall structure provides a passive impediment to invasion, and pathogens must break through this matrix to successfully colonize the cell and cause

infections (Figure 1-2). Plants have a system for monitoring the integrity of the cell wall, in order to trigger defense reactions that lead to dynamic cell wall remodelling required to prevent disease (Bellincampi et al., 2014). Upon pathogen perception, the cell wall is actively transformed and strengthened, specifically at discrete sites of interaction with potentially pathogenic microbes. For example, reinforcement of the cell wall can occur through the deposition of 'papillae' as an early response to perception of numerous pathogens (Underwood, 2012). The rapid deposition of papillae is generally correlated with resistance to fungal pathogens that attempt to penetrate the plant cell wall (Underwood, 2012). Also, the reinforcement of the cell wall with lipid-based polymers that are deposited at the interface between the plant and its environment are important in defense against pathogens. Cuticle containing cutin and embedded waxes coats plant aerial surfaces, and suberin polymers with associated waxes are deposited on the cell wall of various tissues like root endodermis and peridermis (Salminen et al., 2018).

Suberin and cutin are insoluble lipid polymers specific to plants. Both form barriers that restrict transport of water across the cell wall of certain plant tissues, such as the epidermis, endodermis and periderm (Beisson et al., 2012; Vishwanath et al., 2015). They are also known to limit pathogen invasions. While cutin forms the framework of the cuticle sealing the aerial epidermis, suberin is present in the periderm of barks and underground organs. Suberized walls are also found in root internal tissues. Chemical analysis reveals that both polymers are polyesters composed mostly of C16-C18 hydroxyacids, diacids and epoxyacids esterified to each other and to glycerol. However, suberin differs from cutin by having a higher content in C20-C24 aliphatics and phenolic contents (Beisson et al., 2012; Vishwanath et al., 2015). Waxes are associated with both cutin and suberin, and provide

more sealing effects against uncontrolled water movement (Taiz et al., 2014). All plant parts exposed to the atmosphere are coated with the layers of the lipid material.

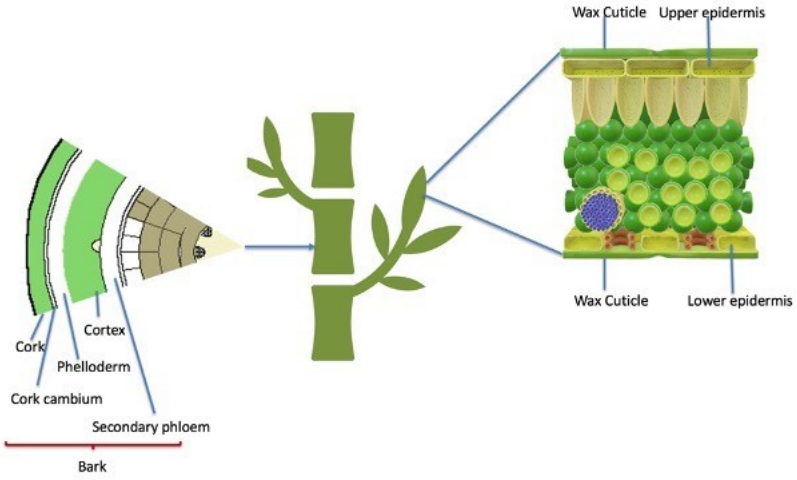


Figure 1-1. Schematic diagram showing the plant's passive outer barriers to pathogens.

Adaptations to prevent pathogen invasion include cuticle and embedded waxes on the leaf surface; intact bark comprised of the cork, cork cambium, phelloderm, cortex and secondary phloem. (Figure modified from Lv et al. (2019)).

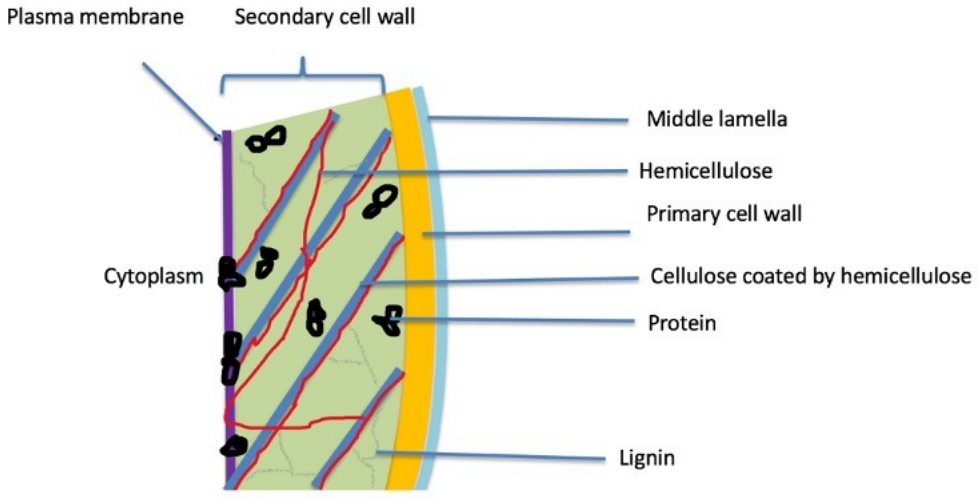


Figure 1-2. Schematic representation of plant cell wall, a barrier against pathogen invasion. (Figure modified from Achyuthan et al. (2010)).

1.5 Localized responses

Plant pathogens that are able to overcome the preformed outer barriers will have to contend with the plant immune system. Infection of plants by pathogens can lead to two distinct outcomes or responses. The first is a localized response in the infected area and the second is a systemic response in non-infected tissues (Figure 1-3). Local immune responses are based on localized surface immune receptors that are able to identify conserved regions of microorganisms called pathogen associated molecular patterns (PAMPs) and this leads to what is known as PAMP or Pattern Triggered Immunity (PTI) (Henry et al., 2013; Spoel & Dong, 2012). Pathogens that inflict damage to plant tissues through cell-wall-degrading enzymes and toxins cause the release of plant cell wall fragments and other components (Gust et al., 2017) which can also act as signaling molecules called damage-associated molecular patterns (DAMPs). DAMPs are recognized by receptors in the plant cell to stimulate immune responses (Carmona et al., 2011; Miedes et al., 2014; Roh & Sohn, 2018).

In turn, plant pathogens have evolved proteins and other specialized macromolecules called ‘effectors’ that can bypass surface receptors and suppress PTI responses from the plant host, and thus facilitate infections. To detect such plant pathogens that have the ability to bypass surface receptors, resistant plants engage what are known as intracellular immune receptors that recognize effector proteins from the pathogens to mount a robust local response known as effector-triggered immunity (ETI). ETI is very effective in preventing pathogen invasion and also provides a mechanism that is able to prime the plant to have enhanced resistance to subsequent attacks by the same pathogen. Apart from the local immune responses produced by ETI, this mechanism is also able to warn distal parts of the plant about invasion by the same pathogen through the production of mobile signals that lead to a systemic response (Henry et al., 2013).

1.6 Systemic responses

A systemic response involves a mechanism whereby an infection of one leaf of a plant leads to local hypersensitive resistance and is immediately followed by the generation of a systemic signal that transfers the same resistance response to other parts of the plant, such that subsequent secondary attacks by the same pathogen are rapidly and effectively blocked (Suzuki et al., 2004). The presence of a pathogen in a plant can thus establish a sort of crosstalk that coordinates responses and enables the plant to 'decide' if the invading pathogen is biotrophic (pathogens that feed and thrive on living host tissues) or necrotrophic (pathogens that kill host tissue before feeding on the remains). The crosstalk between defense responses depends on the levels of major signaling molecules present, and also functions to alert distal tissue of the pathogen detection. This signaling enables the plant to mount an effective defense response against a pathogen (Glazebrook, 2005). Salicylic acid (SA) dependent signaling is the prominent response when biotrophic pathogens are detected by the plant. The presence of SA leads to the activation of known defense genes such as *PATHOGENESIS-RELATED GENE1* (*PR1*). This SA mediated defense mechanism causes a long-lasting systemic response called systemic acquired resistance (SAR). The key regulator of SAR is plant gene *NONEXPRESSER OF PATHOGENESIS-RELATED GENES1* (*NPR1)* (Bigeard et al., 2015; Conrath, 2006; Dong, 2004). However, when necrotrophic pathogens are involved, jasmonic acid (JA) and ethylene (ET) are the molecules used for signaling to induce a defense response that is different from the one induced by biotrophic pathogens (Glazebrook, 2005; Pieterse et al., 2012).

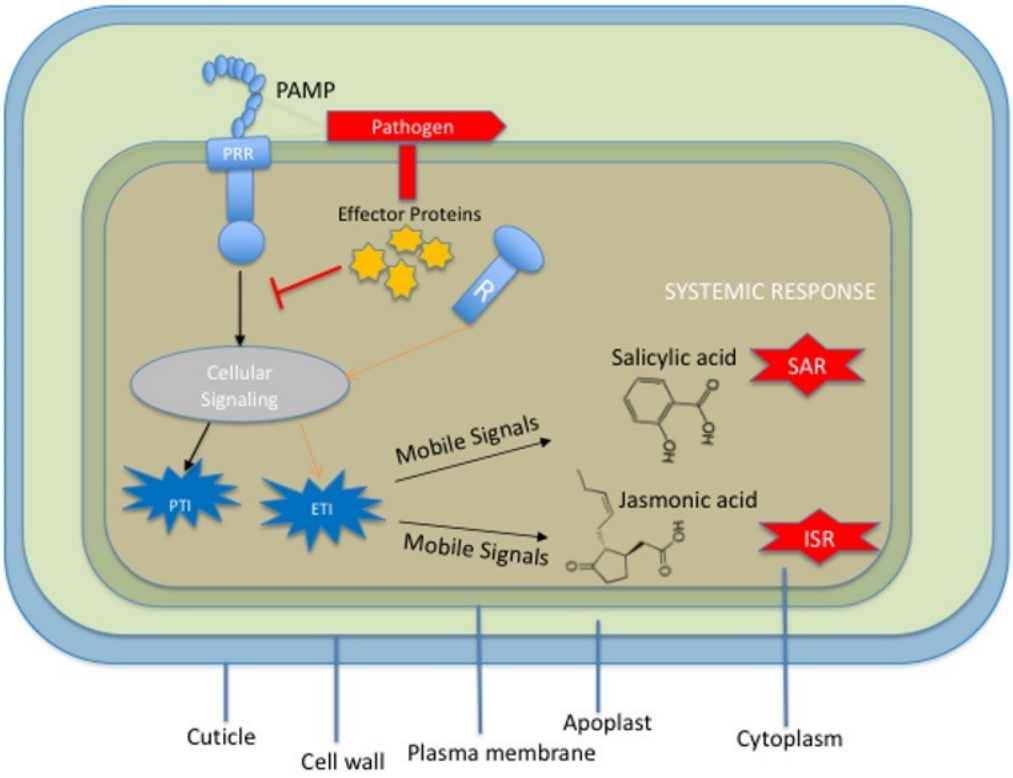


Figure 1-3. Schematic diagram displaying local and systemic responses to pathogens.

From left side, a PTI defense response is triggered by the recognition of PAMPs at the plasma membrane by specialized plant receptors. Pathogens able to secrete effector proteins within the cell that bypass the PTI response can, nevertheless, be recognized by receptors within the plant cell and result in ETI. Mobile signals released following ETI can lead to a systemic immune response depending on the nature of the pathogen. (Figure modified from Dodds & Rathjen (2010)).

1.7 Plant pathogenic microorganisms

Land plants generally live in association with a variety of microorganisms such as viruses, bacteria, fungi, and oomycetes (Teixeira et al., 2019; Zhou & Zhang, 2020). While non-pathogenic microorganisms live in association with plants as commensals or beneficial microbes, pathogenic ones live on plants to obtain nutrients and can disturb mechanisms that are important to plant growth and reproduction. Pathogenicity and virulence are terms that are often used interchangeably to describe pathogenic microorganisms; however, they

have different meanings (Coleman et al., 2011). The capacity of a pathogen to cause disease or damage in a host is referred to as pathogenicity, which is a genetic component of the pathogen, whereas virulence refers to the degree of the disease caused by the pathogen. Pathogens can show a wide range of virulence, usually correlated with the ability of the pathogen to multiply within the host (Pirofski & Casadevall, 2012). Both pathogenicity and virulence can only be manifest in a susceptible host. The ability to differentiate pathogens from commensals or beneficial microorganisms, and properly activate the appropriate defense responses to invading pathogens is a major task for land plants (Zhou & Zhang, 2020). Defense response regulation by the plant is also crucial because defense response activation, in itself, is costly and reduces productivity of the plant (Glazebrook, 2005). When a plant is able to mount a defense against a pathogen and is resistant to such a pathogen, the plant-pathogen interaction is termed an incompatible plant-pathogen interaction, whereas a susceptible plant reaction is called a compatible plant-pathogen interaction (Keen, 1990; Sarmah et al., 2018).

1.7.1 Biotrophic, necrotrophic and hemi-biotrophic pathogens

Plant pathogens are generally classified as biotrophs, hemi-biotrophs, or necrotrophs based on lifestyle and interactions with the host plant (Gebrie, 2016; Chowdhury et al., 2017). Biotrophic pathogens feed on, derive nutrients from, and can survive only on living host tissue. This pattern is in contrast to necrotrophic pathogens, which kill host cells, and feed and grow on the dead and dying tissue. Hemi-biotrophs consecutively use both biotrophic and necrotrophic infection strategies to invade and colonize host plants (Gebrie, 2016; Chowdhury et al., 2017; Lee & Rose, 2010). Hemi-biotrophs are able to shift from

an early biotrophic phase to necrotrophy, and the duration of the biotrophic or necrotrophic phase differs greatly among different hemibiotrophic pathogens (Gebrie, 2016). These different types of pathogens elicit different immune responses by the plant host (Spanu & Panstruga, 2017).

For example, plants employ resistance genes (R genes) and SA signaling in response to biotrophic pathogens. This usually leads to a hypersensitive defense response, which is characterized by rapid cell death at the point of pathogen entrance called Hypersensitive Response-programmed cell death (HR-PCD) (Balint-Kurti, 2019). HR-PCD effectively cuts off the food supply to such pathogens and destroys infected tissues. In contrast, a programmed cell death response to a necrotrophic pathogen would be inappropriate since the host would readily provide nutrition for the pathogen. Thomma et al. (1998) tested Arabidopsis mutants with defects in various defense-related signaling pathways for defects in resistance to various pathogens. The mutation *npr1* and the transgene *NahG*, which block SA signaling, showed loss of resistance to the biotrophic pathogen oomycete *Peronospora parasitica*, but had no effect on resistance to the necrotrophic fungus *Alternaria brassicicola*. In contrast, the *coi1* mutation, which blocks JA signaling, severely compromised resistance to the necrotrophic fungus *A. brassicicola*, but had no effect on resistance to *P. parasitica*. Such observations led to the suggestion that plant defense responses may be tailored to the attacking pathogen, with SA-dependent defenses acting against biotrophs, and JA- and ET-dependent responses acting against necrotrophs (Glazebrook, 2005).

1.7.2 Plant pathogenic Fungi

Plant pathogenic fungi live in or on plant tissues and can cause serious complications for vital physiological functions of the host, and severe losses in crop yields. In order to colonize plant organs, pathogenic fungi use diverse strategies to further invade the host plant and cause disease. An effective pathogenic fungus has to overcome the primary defense systems and cope with a variety of plant defense compounds (Andrade et al., 1999). According to Doehlemann et al. (2017), plant pathogenic fungi are mostly from the Ascomycota and Basidiomycota. For my studies, I primarily focused on the plant pathogenic fungi *Fusarium avenaceum* and *Botrytis cinerea.* Both of these fungi are examples of a filamentous ascomycetes. Species of *Fusarium,* in particular, are among the most economically important plant pathogens (Dean et al., 2012; Rampersad, 2020). In the following section, I provide some background information on these two plant pathogenic fungi

1.7.2.1 *Fusarium* spp.

Included in the *Fusarium* genus of filamentous ascomycetous fungi are many important plant pathogens as well as saprophytes and endophytes (Lysøe et al., 2014). Plant pathogens from the genus *Fusarium*, such as *F. oxysporum*, *F. avenaceum* and *F. graminearum*, may be considered hemi-biotrophs (Perfect & Green, 2001). *Fusarium avenaceum* dwells in the soil, where it forms pale orange sporodochia on the roots of plant hosts, and on agar medium (PDA), the colony is white in surface and reddish orange from the bottom of Petri dishes (Afshari et al., 2020). Like other hemi-biotrophic pathogenic fungi, *F. avenaceum* establishes associations with the hosts by using specialized infection

hypha called haustoria to penetrate into living plant cell wall. Once inside, haustoria expand within the cell and transfer nutrients from the host cell to the fungal thallus (Gebrie, 2016; Koeck et al., 2011; Szabo & Bushnell, 2001). The fungus then proceeds to secrete toxins and enzymes that kill host plant cells to obtain nutrients released from dying tissue. The mechanism of avoiding the host immune system and controlling the living host cells occurs by the production of effector proteins that interact with the host and play an important role in virulence (Koeck et al., 2011). *F. avenaceum* can cause root and stem rots, and vascular wilt diseases in a number of economically important crop species resulting in major yield losses (Chowdhury et al., 2017; Rampersad, 2020).

1.7.2.2 *Botrytis cinerea*

Botrytis cinerea is a necrotrophic fungus that affects many plant species, and it is considered one of the most widespread and common plant pathogenic fungi (Muñoz et al., 2019). *B. cinerea* employs a range of toxic molecules and the plant's own defense systems, such as programmed cell death, to destroy host cells and then feed on the dead cells and to achieve infection (Dean et al., 2012). In order for *B. cinerea* to breach the intact cuticle and invade a plant, it forms dedicated infection structures called appressoria (Ryder & Talbot, 2015). Appressoria are dome-shaped bulges at the ends of germ tubes that develop from spores on the leaf surface of the host plant (Amselem et al., 2011). Appressoria adhere to the leaf surface to facilitate penetration of the fungus into the plant cell.

1.7.3 Plant pathogenic bacteria

A large number of bacterial species are able to cause plant disease. Similar to fungal diseases, bacterial diseases in plants may be characterized by different kinds of symptoms that include galls, wilts, leaf spots, blights, soft rots, as well as scabs and cankers (Figaj et al., 2019). Plant pathogenic bacteria attack plant tissues and often multiply in the extracellular space. Plants have evolved immune responses to recognize and limit the growth of such bacterial pathogens (Lee et al., 2019). To successfully colonize the host and establish infection, some bacterial pathogens have evolved sophisticated systems to combat host defense mechanisms. A recent research survey was conducted among bacterial pathologists to ask them to nominate the bacterial pathogens they would place in a 'Top 10' based on importance to science and impact on food production and/or the environment. The survey generated 458 votes from the international community and *Pseudomonas syringae,* used in my studies, was at top of the list (Mansfield et al., 2012).

1.7.3.1 *Pseudomonas syringae*

P. syringae is a versatile bacterial plant pathogen that is present worldwide and lives primarily as an epiphytic, opportunistic pathogen on plant surfaces (Martins et al., 2018; Romantschuk et al., 1997). *P. syringae* is divided into more that forty pathovars (pv) based on host specificity (Fouts et al., 2003). For instance, *P. syringae* pv. *tomato* infects and causes 'bacterial speck disease' in plants such as tomato and Arabidopsis, but it elicits the defense-associated hypersensitive response (HR) in bean, tobacco, and many other plants. The economic impact of *P. syringae* cannot be overemphasized and is increasing (Mansfield et al., 2012). Studies with *P. syringae* lead the field in using high-throughput

sequencing technologies to understand pathogenicity (Mansfield et al., 2012). *P. syringae* was first shown in the 1980s to infect Arabidopsis and also produce symptoms in the laboratory environment (Dong et al., 1991). The researchers did this through testing many *P. syringae* strains on various Arabidopsis accessions (Dong et al., 1991; Katagiri et al., 2002), and the two virulent strains most extensively used in research today, *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *maculicola* ES4326, initiated from this research.

P. syringae gains entrance into the host tissues (mainly leaves) via wounds and natural openings like the stomata. Within the intercellular spaces, the pathogen rapidly proliferates if the plant is susceptible and produces what appear as water-soaked patches in infected leaves. However, if the plant is resistant, a hypersensitive response (HR) by the plant is triggered (Dong et al., 1991; Katagiri et al., 2002). Research into the molecular biology of virulence and plant defense against *P. syringae* has opened up new insights into microbial pathogenicity. Discoveries concerning the effector trafficking and host targets for defense suppression to facilitate infection are notable examples (Mansfield et al., 2012), which were made possible because the pathogen carries a range of virulence factors known as the type III secretion system (T3SS) effector proteins. A 23-kb hrp (hypersensitive response and pathogenicity) gene cluster is the major source of T3SS effectors in *P. syringae* (Ichinose et al., 2013). Among the proteins encoded by the hrp gene cluster, some are also conserved in other plant and animal pathogenic bacteria and were renamed hrc (hrp conserved) and are thought to encode the core components of the secretion apparatus (Weber et al., 2005). *P. syringae* uses the type III secretion system by injecting effector (Avr or Hop) proteins into plant cells. In susceptible hosts, these cause effector triggered susceptibility (ETS), and the induction of the hypersensitive response in resistant plants

leading to effector triggered immunity (ETI) (Fouts et al., 2003). Some Hrp effector proteins are termed Avr because of the avirulence (lacking competence to produce pathologic effects) phenotype they confer on races of a given pathovar when tested in cultivars of the host species that carry related resistance (R) genes (Dangl & Jones, 2001; Fouts et al., 2003). Mutation in the hrp conserved gene cluster leads to *P. syringae* pathovar (pv.) tomato DC3000 known as *hrcC*$^{-}$ (*Pst hrcC*$^{-}$*),* a mutant that is defective in the Hrp secretion machinery and so unable to elicit the HR response in nonhosts or be pathogenic in host plants because it cannot deliver T3SS-dependent effectors (T3SEs) into plant cells (Hauck et al., 2003). Because of this, *Pst hrcC*$^{-}$ is unable to disrupt PAMP detection to cause ETI. Consequently, PTI is the main immune response against infection and colonization of Arabidopsis by *Pst hrcC*$^{-}$ (Ichinose et al., 2013; Mansfield et al., 2012).

1.8 Innate immunity

Plant disease resistance may be determined by a plant's innate immune system which is classified by the type of immune receptor, which recognize the exact ligands to activate defense (Jones & Dangl, 2006; van Wersch et al., 2016; Zipfel & Robatzek, 2010). The first line of immune response is Pathogen Associated Molecular Pattern (PAMP) - triggered immunity (PTI) or basal defense. The second is known as effector-triggered immunity (ETI). PTI recognizes and responds to molecules common to many microbes, including non-pathogens (Jones & Dangl, 2006), and is triggered by the activities of surface membrane-anchored pattern recognition receptors (PPRs) that are localized to the plasma membrane of the plant's cell surface. PPRs are able to recognize and bind PAMPs, conserved patterns of pathogens. Some pathogens have the capability to bypass PTI by using the Type III secretion system (T3SS) (De Wit, 2007; van Wersch et al., 2016).

Through the T3SS, the pathogen releases directly into host plant cells effectors that supress PTI and thereby lead to the activation of what is known as effector-triggered susceptibility (ETS). As means of counteracting this pathogen virulence tactic, the plant in turn switches to a second level which works in elicitor-mediated defenses and includes receptors that are cytoplasmic proteins able to detect strain-specific pathogen effectors (van Wersch et al., 2016). The plants produce resistance proteins (R) intracellularly, which respond to pathogen virulence factors, either directly or through their effects on host targets, and are able to recognize the pathogen effector protein and bind to it, thereby leading to the activation of ETI (Jones & Dangl, 2006).

ETI is basically an amplified PTI response, as it involves PTI transcriptional programs and antimicrobial defenses and is also characterized by hypersensitive reaction that leads to cell death at the site of infection and localized hormone production resulting in defense gene activation (Dangl et al., 2013). Activation of ETI often results in generation of signal molecules including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). Salicylic acid–dependent signaling is foremost in response to biotrophic pathogens and leads to the activation of defense effector genes including *PATHOGENESIS RELATED1 (PR1).* Activation of the SA pathway induces what is known as systemic acquired resistance (SAR). SAR is a secondary immune response in the distal parts of plants, which causes plant tissues to become resistant to a broad range of pathogens for a long period of time. Also, SAR allows for defense responses that are enhanced and faster during subsequent attacks (Freeman & Beattie, 2008; Dangl et al., 2013). Jasmonic acid and ethylene signaling pathways are major players in response to necrotrophic pathogens and

chewing insects (Dangl et al., 2013). JA signaling also induces what is known as induced systemic resistance (ISR) after root exposure to beneficial soil microbes.

Innate immunity appears to be a crucial development in the colonization of land by plants. It evolved in a common ancestor of mosses, liverworts and hornworts, which are among the first land plants (Ponce de León & Montesano, 2017; Uehling et al., 2017). For example, the hornwort *Physcomitrella patens* recognizes PAMPs via plasma membrane receptors, and using a MAP kinase (MAPK) cascade, the recognition leads to the activation of cell wall associated defenses similar to those in flowering plants (Uehling et al., 2017).

In the next section, I provide more information on the key players in innate immunity: PAMPS, effectors and PRRS.

1.8.1 Pathogen associated molecular patterns (PAMPs)

PAMPs are conserved regions of molecules shared among microorganisms that signal pathogen presence to host cells that can then defend against infection. Common PAMPs include bacterial flagellin, bacterial elongation factors, peptidoglycan, and lipopolysaccharides (van Wersch et al., 2016; Zipfel & Robatzek, 2010). When pathogens come in contact with a plant, cells secrete lytic enzymes to release PAMP epitopes from the attacking pathogens, as in the case of the bacterial flagellin epitope flg22 (Buscaill et al., 2019; Liu et al., 2014; Zhou & Zhang, 2020)**.** PAMPs are recognized by pattern recognition receptors (PRRs) proteins located on the plasma membrane, and the detection of PAMPs by PRRs elicits a series of signaling events and transcriptional reprogramming that includes plant defense responses and deters further spread of the pathogen. This process is called PAMP or pattern-triggered immunity – PTI (Figure 1-4). PTI responses

include, but are not limited to, the production of reactive oxygen species (ROS) within minutes of perception (Doehlemann & Hemetsberger, 2013), calcium influx, alterations and reinforcement of the cell wall by deposition of callose, and the accumulation of defense hormones (Shamrai, 2014).

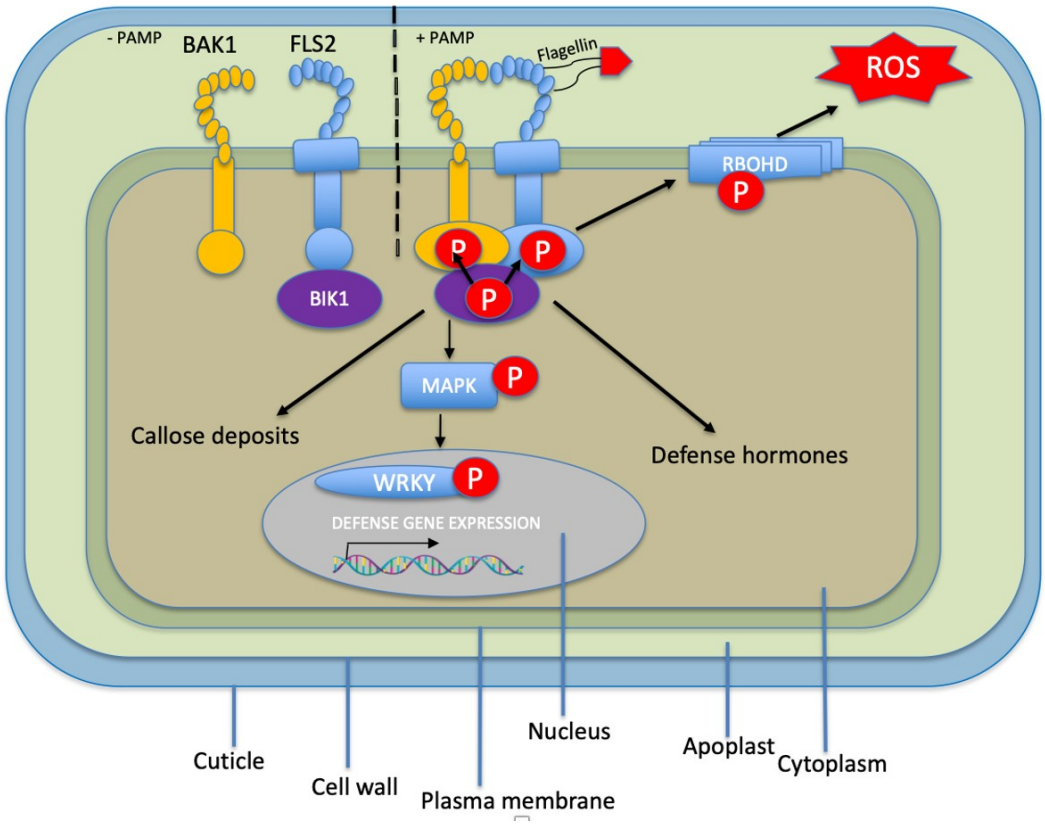


Figure 1-4. Schematic diagram depicting basic PTI signaling pathway triggered by bacterial flagellin.

At top left, the FLS2 receptor in inactive state interacts with BIK1. When pathogen or PAMP (flg22 peptide) is present, FLS2 interacts with co-receptor BAK1 to form a stable heterodimer that activates BIK1 phosphorylation. BIK1 phosphorylates both BAK1 and FLS2 leading to downstream activation of a MAP kinase (MAPK) phosphorylation signaling cascade resulting in expression of defense genes, callose deposition and defense hormone production. RBOHD is also phosphorylated by BIK1 causing a rapid transient production of reactive oxygen species (ROS) in the apoplast. P = phosphate group. (Figure modified from Dodds & Rathjen (2010)).

1.8.2 Plant Pattern Recognition Receptors (PRRs)

PRRs are proteins that are able to bind specifically to conserved portions of PAMPs. All plant pattern recognition receptors are surface-localized ligand-binding receptors, which can interact with PAMPs on the surface of the invading pathogen. This is quite different from mammal PRRs, that employ both intracellular and surface localized PRRs to perceive PAMPs and DAMPs (Amarante-Mendes et al., 2018; Macho & Zipfel, 2014) (Figure 1-4). Plant PRRs are either receptor-like kinases (RLKs), or receptor-like proteins (RLPs), which do not exhibit any known intracellular signaling domain (Boutrot & Zipfel, 2017). According to Saijo et al. (2018), RLKs are made up three components, including an extracellular domain (ectodomain), transmembrane domain and an intracellular kinase domain, while RLPs have only two components, the extracellular domain (ectodomain), and transmembrane domain, but lack the intracellular kinase domain. Ectodomain classes largely match to the nature of the ligands recognized. Ligand-binding ectodomains described to date include, leucin rich repeats (LRRs), lysin motifs (LysMs), lectin-like motifs (LLMs) and epidermal growth factor (EGF)-like domains.

Different PRRs and the matching PAMPs are known, including: Arabidopsis FLAGELLIN SENSITIVE2 (LRR-RLK FLS2) receptor that recognizes an N-terminal 22-amino-acid epitope (flg22) of bacterial flagellin (Zhang et al., 2017); ELONGATION FACTOR TU RECEPTOR (LRR-RLK EFR) which recognizes an N-acetylated 18–26-amino-acid epitope (elf18–elf26) of elongation factor thermo unstable (Tu) (Boutrot & Zipfel, 2017; Zhang et al., 2017); and rice *Xanthomonas* resistance protein 21 (XA21) (Dardick & Ronald, 2006). FLS2, EFR and XA21 all belong to the leucine rich repeats-receptor-like kinases (LRR-RK) class (Saijo et al., 2018; Shiu et al., 2004), and offer

excellent PRR models. Arabidopsis FLAGELLIN SENSITIVE2 (FLS2) receptor, which recognizes the N-terminus of 22-amino-acid epitope (flg22) of bacterial flagellin from the bacteria *Pseudomonas aeruginosa* is the best studied PRR and PAMP (Bigeard et al., 2015; Gómez-Gómez & Boller, 2000; Macho & Zipfel, 2014).

As stated by Gou et al. (2012) and Zhou & Zhang (2020), it is well known that ligand perception by PRRs requires co-receptors (Figure 1-4). For example, in the FLS2 receptor pathway, FLS2 activity depends on the SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) family of LRR-RKs such as the BRASSINOSTEROID INSENSITIVE1-ASSOCIATED RECEPTOR KINASE1 (BAK1) co-receptor to initiate the signaling. BAK1 interaction with FLS2 is brought about by flg22. In order to send phosphorylation signals from the plasma membrane to the nucleus, receptor-like cytoplasmic kinases (RLCKs) that can associate with the plasma membrane and are directed substrates of the ligand-binding receptor complexes are involved. For FLS2, BOTRYTIS-INDUCED KINASE1 (BIK1) is a signaling component in the pathways (Bücherl et al., 2017), which is promptly phosphorylated and released from the receptor complex once a pathogen is detected (Bigeard et al., 2015).

1.8.3 Cellular and physiological responses triggered by pattern recognitions

Response outcomes brought about by PRR perception of PAMPs are mediated by a number of kinases that play critical roles in linking PRRs to key downstream signaling modules thereby coordinating cellular and physiological responses in plants (Yu et al., 2017). On the plasma membrane and the cytoplasm of the plant cells, an FLS2-flg22 interaction triggers a number of key signaling responses including the rapid

phosphorylation of receptor-like cytoplasmic kinases (RLCKs) and activation of MAPK cascades, an influx of calcium across the plasma membrane, the transient production of an H_2O_2 oxidative burst, and the activation of calcium-dependent kinases (CPKs), all leading to the upregulation of defense genes, deposition of callose in cell walls, and enhanced resistance against pathogens (Danna et al., 2011; Zhou & Zhang, 2020). Different cellular responses and regulations upon pattern recognition represent hallmarks of PTI elicitation in the plant, which collectively contribute to plant resistance against a broad spectrum of pathogens. These responses occur within seconds to minutes or as late as hours to days (Yu et al., 2017), once a pathogen is detected. Such responses are generally categorized into early or late responses.

1.8.3.1 Early responses

Among the early responses triggered by the perception of PAMPs by PRRs are ion fluxes, protein phosphorylation, reactive oxygen species (ROS) generation, and other signal transduction events (Robatzek, 2014). According to Nurnberger et al. (2004), the ion flux events of plant responses to microbe associated molecular patterns (MAMPs) occurred within approximately 0.5–2 min. This ion flux includes increased influx of Ca^{2+} and efflux of K^+ and NO_3^-, which results in a rapid and transient extracellular alkalinisation and depolarization of the plasma membrane and an increased cytosolic Ca^{2+} (Yu et al., 2017; Zipfel & Robatzek, 2010). An influx of Ca^{2+} from the apoplast that leads to a rapid increase of Ca^{2+}concentrations in cytoplasm may activate calcium-dependent protein kinases (Ludwig et al., 2005). According to Tang et al. (2017), MAMP-triggered calcium influx is of crucial importance to plant immunity.

In Arabidopsis, transient and rapid generation of apoplastic (extracellular space) reactive oxygen species (ROS), referred to as a 'ROS burst', is a hallmark of PTI and represent an early response to PAMP detection (Torres et al., 2006). The kinase BIK1 phosphorylates and activates downstream components of the ROS burst. For instance, BIK1 directly phosphorylates the N terminus of NADPH oxidase respiratory burst oxidase homolog D (RbohD) to stimulate ROS (Zhou & Zhang, 2020). This phosphorylation occurs at around 2-3 minutes after pathogen perception. An ROS burst is brought about by both plasma membrane-localized NADPH oxidases and cell wall–associated peroxidases induced by PAMPs. The NADPH oxidase involved in MAMP-induced ROS production is also RBOHD (Nühse et al., 2007; Ranf et al., 2011; Yu et al., 2017) such that a MAMP-induced ROS burst is strikingly compromised in the *rbohD* mutant (Nühse et al., 2007). NADPH oxidases transfer electrons from cytosolic NADPH to apoplastic oxygen, leading to the production of O_2^- in the apoplast, which is membrane impermeable but rapidly converted to H_2O_2 by superoxide dismutase allowing entry to the cytosol where it can play a signaling role (Ranf et al., 2011; Yu et al., 2017). H_2O_2 also acts a toxic barrier to subsequent pathogen infections, involved in strengthening plant cell walls by oxidative cross-linking of polymers (Ranf et al., 2011; Torres et al., 2006; Yu et al., 2017).

Other PTI early responses are signal transduction events by several kinases and phosphatases proteins. Calcium-dependent protein kinases (CDPKs) function in transcriptional reprogramming, while mitogen-activated protein kinase (MAPKs) help translate extracellular stimuli into appropriate intercellular responses (Bigeard et al., 2015). Arabidopsis genes code for 20 MAPKs, 10 MAPKKs, and 60 MAPKKKs. Many PTI signaling modules are initiated by four MAPKs: MAPK3, MAPK4, MAPK6 and MAPK11

(Bigeard et al., 2015; Melotto et al., 2006). Melotto et al. (2006) reported that stomatal closure is part of an integral innate immune system that acts as a barrier against bacterial infection. Thus, stomatal closure is another aspect of a plant innate immune response that is initiated within the first hour of pathogen perception. The stomata consist of a pair of guard cells (specialized epidermal cells) that regulate gas exchange and water loss and also serve as a natural opening to invading pathogens. Stomatal guard cells of Arabidopsis perceive bacterial surface molecules, which involves the FLS2 receptor, and one hour after perception, stomata begin to close (Melotto et al., 2006). PAMPs can trigger stomata closure in a SA-dependent manner (Shen et al., 2017).

1.8.3.2 Late responses

Genes involved in late responses include marker defense genes such as *PR1,* defense genes specific to the PTI pathway like *FLG22-INDUCED RECEPTOR-LIKE KINASE1 (FRK1)* and *ARABIDOPSIS NON-RACE SPECIFIC DISEASE RESISTANCE/TOBACCO HAIRPIN-INDUCED GENE-LIKE10 (NHL10),* genes involved in antimicrobial compound synthesis, callose biosynthetic genes and others (Bigeard et al., 2015). As an interesting example, it was shown that genes associated with photosynthesis-related processes were considerably downregulated as a late response to PAMP perception (Uehling et al., 2017). This suppression of photosynthesis leads to the reduced availability of resources and nutrients for both the plant and the pathogen, serving as a mechanism to prevent further microbial growth or colonization (Bo Li et al., 2016) and reduce the growth of the plant (Huot et al., 2014). Another example of a late response of PTI immune signaling is the reinforcement of the outer barriers to pathogen invasion. This response

involves the accumulation of several polymers in areas of pathogen attack. Notable is the deposition of papillae containing the β 1,3-glucose polymer callose, which is deposited at sites of injury in the cell wall (Hématy et al., 2009). The late response gene, *GLUCAN SYNTHASE5 (GSL5),* is a callose synthase in Arabidopsis (Ellinger & Voigt, 2014; Jacobs et al., 2003). Callose synthases form callose using UDP-glucose as a substrate beginning around 12 hours following the perception of pathogen in Arabidopsis (Li et al., 2016).

1.9 Effector triggered immunity

The immune response triggered by effectors, also known as R gene–mediated resistance, is considered the second line of plant defense. This response involves a race specific hypersensitive reaction (HR) that leads to cell death. Hence, pathogen growth is suppressed by effector-triggered downstream genes. HR is a hallmark of ETI (Ajjamada et al., 2016). Effector perceptions by NLRs leads to immune responses similar to the ones triggered by PAMP perception via PRRs, including the massive production of ROS, phosphorylation of a number of immune signaling, calcium influxes, MAP kinase activation, expression of defense genes, synthesis of defense hormones, and programmed cell death (Zhou & Zhang, 2020). The major differences between ETI and PTI are that while activation of NLR (ETI response) triggers programmed cell death, activation of PRRs (PTI response) usually does not trigger cell death. The initiation of an ROS burst, calcium influx, and MAPK cascade by PRRs is very transient in nature, whereas these responses after NLR activation are often delayed and prolonged (Peng et al., 2018; Su et al., 2018; Zhou & Zhang, 2020). Some recent studies suggested that activation of NLRs by transgenic expression of cognate effectors only induced defenses moderately in the absence of MAMP perception and that concurrent prompting by PRRs and NLRs is required for the

full activation of defenses including ROS burst, callose deposition, prolonged MAPK activation, defense gene induction, HR, and complete disease resistance (Zhou & Zhang, 2020). It was observed that pre-activation of NLRs also primes PRR-mediated defenses suggesting close crosstalk between the PRR and NLR pathways. R gene–mediated resistance also involves activation of a salicylic acid (SA)-dependent signaling pathway (in biotrophic pathogens), that primes expression of certain pathogenesis-related (PR) proteins thought to contribute to resistance. Plant defense responses against necrotrophs are controlled by mechanisms dependent on ethylene (ET) and/or jasmonates (JA) (Sarmah et al., 2018).

1.9.1 Effectors

Effectors are intracellular elicitors (protein molecules) encoded by specific avirulence (AVR) genes of certain pathogenic microorganisms in an effort to overcome PTI (Ajjamada et al., 2016; Boller & Felix, 2009). Effector proteins differ from PAMPs in some ways; while PAMPs are recognized by ligand binding pattern recognition receptors (PRRs) that are either RLKs or RLPs located on the plasma membrane, effectors are perceived by the plant's intracellular resistance proteins (R proteins), which are nucleotide-binding leucine rich repeat (NLR) receptors (Dangl et al., 2013; Zhou & Zhang, 2020). Therefore, any given infection involves the perception of multiple danger signals by different immune receptors (Zhou & Zhang, 2020). The detection of pathogen effectors by NLR receptors elicits an ETI response, which is a series of multifaceted networked signaling events and transcriptional reprogramming (Jones & Dangl, 2006; Selin et al., 2016; Zhou & Zhang, 2020). Effector recognition receptor genes can be considered as

surveillance genes in contrast to “real” resistance genes that induce hypersensitive response or callose synthase (Dodds & Rathjen, 2010). Effectors are considered to be specific to biotrophic pathogens, however, according to Boller & Felix (2009), are also produced by several necrotrophs.

1.9.2 Hypersensitive reaction

A hypersensitive response (HR) in plants is usually triggered by the perception of biotrophic plant pathogens including fungi, bacteria, and viruses (Dangl & Jones, 2001). HR is produced by rapid local changes that lead to the generation of reactive oxygen species (ROS) which cause programmed cell death in the local area around the infection with characteristic visible lesions. This mechanism is employed by plants to limit the growth and spread of pathogens to other parts of the plant. The necrotic plant cells restrict further pathogen development and may also act as reservoirs to gather metabolites that are toxic to the pathogen (phytoalexins) that may also serve as secondary elicitors that can stimulate defense in surrounding plant cells.

1.9.3 Defense hormones in plants (phytohormones)

Phytohormones are small molecules that are produced in one area/part of a plant that can travel and act on other parts of the plant to effect physiology, such as growth, development and stress responses, including defense against pathogens (Provart et al., 2016). In particular, the roles of phytohormones in plant defense cannot be overemphasized. The main hormones implicated in plant innate immunity against pathogens are salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Bigeard et al., 2015). These hormones

are generally produced at the site of attack and can result in specific outcomes directed towards the invading pathogen. It has been established that SA plays essential roles in resistance against biotrophic and hemibiotrophic pathogens such as *P. syringae,* whereas JA/ET signaling confers resistance to necrotrophs (Glazebrook, 2005; Pieterse et al., 2012). Amplification of both PRR- and NLR-mediated immune signaling requires SA (Zhang & Li, 2019). SA binds to NPR1 as an important step in the defense response; this binding activates NPR1 to promote the expression of downstream defense genes (Ding & Ding, 2020) and can lead to a longer-lasting systemic acquired resistance (SAR) response (Fu & Dong, 2013; Zhou & Zhang, 2020). When Arabidopsis mutants that are deficient in SA (e.g., *salicylic acid-deficient2*) were analyzed, it was found that pathogen-induced SA is synthesized through the isochorismate pathway as in some bacteria (Zhang & Li, 2019). In contrast to SA, JA and ET signaling pathways are important in defense against necrotrophic pathogens and chewing insects (Dangl et al., 2013). JA signaling also induces what is known as induced systemic resistance (ISR) after root exposure to beneficial soil microbes.

1.10 Role of BTB-ankyrin proteins in defense

BTB-ankyrin proteins are plant-specific transcriptional co-activators discovered in Arabidopsis that play roles in the regulation of both development and defense (Khan et al., 2014). BTB-ankyrin proteins are characterized by two conserved protein-protein interaction motifs found in all members of the family: a BTB/POZ (for Broad Complex, Tram track, and Bric-a-brac/POX virus and Zinc finger) domain at the N-terminus and four ankryin motifs near the C-terminus (Cao et al., 1997; Hepworth et al., 2005). Members of this protein family have a transcriptional activation domain, but lack a DNA-binding domain, and therefore employ the activities of TGACG motif-binding (TGA) basic leucine

zipper (bZIP) proteins as co-factors for recruitment to DNA (Fu & Dong, 2013). While the BTB/POZ domain functions to facilitate dimerization and interacts with CULLIN3 to target proteins for degradation (Chahtane et al., 2018; Zhang et al., 2017), the ankyrin repeats interact with basic leucine zipper (bZIP) transcription factors TGA (TGACG-motif binding) that bind to DNA (Després et al., 2000).

The Arabidopsis genome encodes six BTB-ankyrin proteins. Phylogenetic analysis classifies the six members into a primary subclade including NON-EXPRESSER OF PATHOGENESIS RELATED GENE 1 (NPR1) and its three closest homologs (NPR2, NPR3, and NPR4) with roles in plant defense; and a secondary subclade comprising BOP1 and BOP2. The expression of BOP1 and BOP2 are generally restrained in lateral organ boundaries (LOBs) and are described to have an important role in plant development, particularly in leaf and inflorescence architecture (Fu et al., 2012; Hepworth et al., 2005; Khan et al., 2014; Zhang et al., 2019) (Figure 1-5A). Analysis of genomic data suggested that BTB-ankyrin proteins originated before land plants appeared, and homologs of both NPR1 and BOP1/2 are encoded by mosses and higher land plants, suggesting that the ancestral BTB-ankyrin proteins may have been involved in both defense and development (Khan et al., 2014; Lewis & McCourt, 2004; Zhang et al., 2019). The Arabidopsis genome encodes ten TGA factors subdivided into five clades: clade I includes TGA1 and TGA4 (TGA1/4); clade II members are TGA2, TGA 5, and TGA 6; clade III contains TGA3 and TGA7; clade IV includes TGA9 and TGA10; and clade V comprises TGA8/PERIANTHIA (Gatz, 2013) (Figure 1-5B).

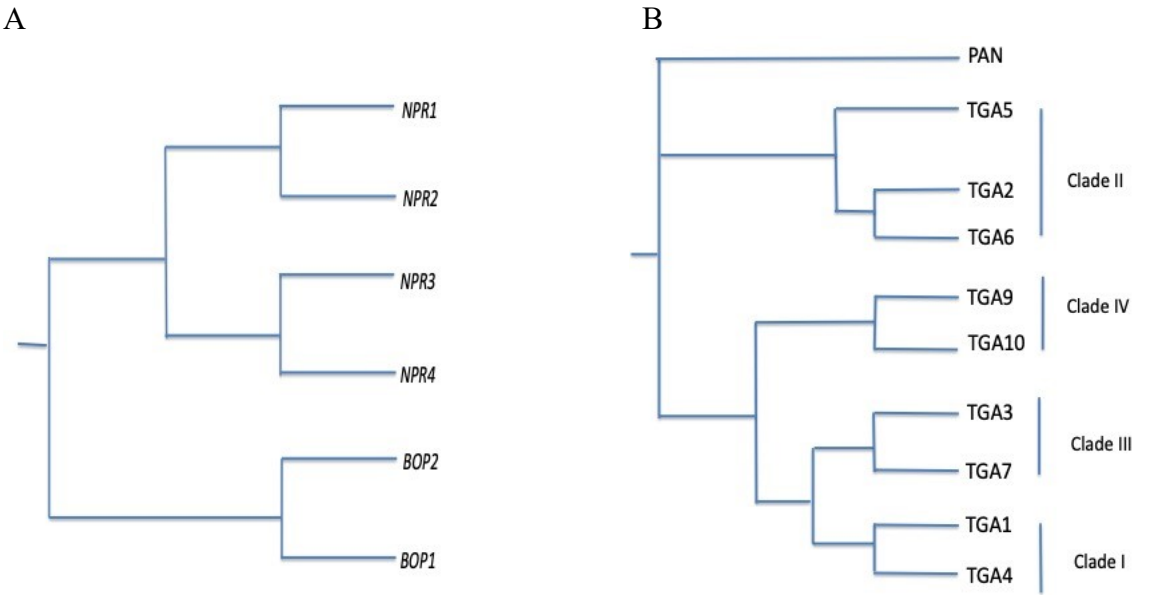


Figure 1-5. Phylogenetic relationship of BTB-ankyrin proteins and TGA transcription factors.

(A) Analysis of BTB-ankyrin proteins from Arabidopsis showing that members are closely related but for three distinct pairs. Arabidopsis Genome Initiative numbers are as follows: NPR1, At1g64280; BOP1, At3g57130; and BOP2, At2g41370. (B) Arabidopsis TGA transcription factor family. Arabidopsis Genome Initiative numbers are as follows: PAN, At1g68640; TGA5, At5g06960; TGA2, At5g06950; TGA6, At3g12250; AtbZIP21, At1g08320; AtbZIP65, At5g06839; TGA3, At1g22070; TGA7, At1g77920; TGA1, At5g65210; TGA4, At5g10030. (Figure modified from Hepworth et al. (2005)).

1.10.1 NON-EXPRESSER OF PATHOGENESIS RELATED GENES 1 (NPR1) regulates defense

NPR1 was the first BTB-ankyrin protein to be characterized and is essential for the regulation of systemic acquired resistance (SAR), a long-lasting disease resistance response that involves the up regulation of pathogenesis-related (PR) genes in Arabidopsis (Fu & Dong, 2013). A function for NPR1 in plant defense against pathogenic infection was first identified based on loss-of-function mutations that eliminated SAR (Cao et al., 1997; Cao Hui et al., 1994). NPR1 interacts constitutively with Clade II TGAs, and in a redox-dependent manner with Clade I TGAs (Després et al., 2000; Després et al., 2003) . After

local infection, there is an increase in the levels of SA hormone, which prompts the enzymatic reduction of NPR1 to a monomeric state, making it possible for the protein to translocate from the cytoplasm into the nucleus (Mou et al., 2003). While in the nucleus, NPR1 interacts with various TGA bZIP transcription factors, allowing the induction of gene expression to induce SAR (Zhou et al., 2000). Zhang et al. (2003) showed that knockout mutants of TGA2, TGA5, and TGA6 show defects in SA-dependent gene expression and disease resistance similar to *npr1* mutants. This result indicated that clade II TGAs interact with NPR1 and play a major role in SAR. NPR1 interactions also occur with clade III TGA3 (Choi et al., 2010), and with reduced forms of clade I TGA1 and TGA4 to regulate SA-dependent immunity (Després et al., 2003; Sun et al., 2018).

1.10.2 Plant defense against pathogens versus plant development

Defense activation mechanisms in plants are essential for the combat of pathogens. However, this activation is resource costly and occurs at the expense of growth (Huot et al., 2014; Karasov et al., 2017; Neuser et al., 2019). Plants that unnecessarily activate defense responses can experience severe reductions in growth that impair reproductive fitness (Huot et al., 2014). Autoimmune mutants are a class that perpetually express defense genes. These mutants exhibit broad-spectrum resistance to pathogens combined with growth retardation and frequently, spontaneous cell death. Other features of auto-immune mutants include the elevation of defense hormones (SA, JA), elevated resting ROS levels, and the accumulation of callose deposits in leaves (van Wersch et al., 2016). Plants that overexpress BOP1 or BOP2 display a dwarf phenotype and exhibit late flowering with

elevated levels of JA (Khan et al., 2015; Norberg et al., 2005). These features suggest a possible autoimmune phenotype.

1.11 Suberin polymer

As mentioned, late responses to pathogen interactions can result in structural changes throughout the plant. These structural changes can include reinforcement of the cell wall with lipid-based polymers and other secondary metabolites (Salminen et al., 2018). Suberin is a protective polymeric barrier that is naturally deposited in the cell wall of certain plant tissues, including root endodermis, root and tuber peridermis, and seed coats (tissue–tissue or plant–environment interfaces) (Wei et al., 2020). Suberin with its associated waxes forms a physiologically important hydrophobic barrier affecting water and solute transport, nutrient and gas exchange, toxic chemical uptake and may limit pathogen invasion (Graça, 2015; Kosma et al., 2014; Wei et al., 2020). Suberin deposition may be induced to either increase the amount of suberin in previously suberized cell walls or initiate suberization non-suberized cell walls. For example, suberin deposition is induced in response to wounding and pathogen attack (Domergue et al., 2010), and salt stress (Barberon et al., 2016; Kosma et al., 2014). It is believed that plants synthesize and deposit suberin whenever they need to form a persistent barrier (Franke & Schreiber, 2007; Vishwanath et al., 2015).

1.12 Chemical composition of suberin

According to Bernards (2002) and Pollard et al. (2008) suberin is a complex lipophilic heteropolymer made up of aliphatics, glycerol and aromatic components, and is associated with some soluble waxes. Ester breaking reactions have been used to

depolymerize suberin, and the major building block units are shown to be interlinked as a polyester macromolecule (Graça, 2015; Wei et al., 2020). Quantification of suberin monomer composition depolymerized by base- or acid-catalyzed depolymerization shows methyl esters of the monomers that can be identified by gas chromatography-mass spectrometry (GC-MS) and quantified by GC-MS or GC-flame ionization detection (GC-FID) (Ranathunge & Schreiber, 2011).

The major components of depolymerized suberin of *A. thaliana* are long-chain aliphatic acids, representing about 80–90% of the mass of all monomers released (Graça, 2015). Suberin monomers are the aliphatics that are mainly of long-chain (C16 and C18) and very-long-chain (≥C20), including ω-hydroxy fatty acids (OH-FAs), α, ω-dicarboxylic fatty acids (DCAs), unsubstituted fatty acids (FAs), and primary fatty alcohols and diols (Graça & Santos, 2007; Schreiber et al., 1999). Suberin aliphatics are known to play physiologically important roles in the suberin properties of water sealing and fungal resistance (Lulai & Corsini, 1998; Wei et al., 2020). Another main constituent of the poly-aliphatic component is glycerol, which has been shown to function to link together different fatty acyl types and hydroxyl groups via esterification processes (Andersen et al., 2015). *p*-hydroxycinnamic acid derivatives, especially ferulate, are the main constituents of the aromatic component (Kolattukudy, 2001) (Table 1-1). In Arabidopsis, suberin polymer is assumed to be embedded with waxes (Delude et al., 2016; Kosma et al., 2012), and such waxes are non-polymeric, solvent-soluble, lipophilic compounds which are believed to be a major contributor to the barrier function of suberin in roots (Ranathunge & Schreiber, 2011). Suberin-associated waxes have been extracted by brief immersion of tissues in chloroform (Kosma et al., 2012; Vishwanath et al., 2013), or by extensive solvent

extraction methods (Delude et al., 2016). Phenolics are also released, including ferulic acid, when suberin is depolymerized (Graça, 2015). In Arabidopsis roots, according to Delude et al. (2016) and Vishwanath et al. (2013), suberin polymer and associated waxes biosynthesis occurs in parallel and involves common pathways.

Despite the fact that the monomeric composition of suberin polymer is known for many plants, the flow of biosynthetic reactions, transport mechanism of monomers, and polymerization, are still not very clear (Vishwanath et al., 2015). However, many genes encoding enzymes involved in the biosynthesis of suberin have been identified in Arabidopsis and potato, with the aid of many quantitative analytical tools developed to measure suberin components after depolymerization, coupled with characterization of many Arabidopsis mutants with altered suberin composition (Vishwanath et al., 2015).

1.13 Suberin structure

Due to difficulty in isolating unaltered intact suberin, the exact macromolecular organization of suberin polymers remains to be fully elucidated. However, a suberin structural model has been proposed based on the chemical composition and data generated by partial depolymerization of tree bark (cork) and tuber periderms (Bernards, 2002; Graça, 2015; Ranathunge & Schreiber, 2011). Transmission electron microscopy (TEM) reveals the ultrastructure of suberized cell walls as two types of alternating lamellae, the “light,” electron translucent lamellae (suberin poly-aliphatic domain (SPAD)), and the “dark,” electron-opaque ones (suberin poly-phenolic domain (SPPD)) (Bernards, 2002). Suberin is structurally and chemically similar to cutin, which is contained in plant cuticles. Cutin is comprised of an aliphatic acylglycerol-based polyester matrix, but in contrast to suberin, cutin is accumulated on the outer surface of the epidermal cell wall of aerial plant organs,

whereas suberin is deposited on the inner face of the cell wall adjacent to the plasma membrane (Franke et al., 2012; Vishwanath et al., 2015).

Suberin monomers are also very similar to cutin monomers, although longer chain lengths and greater proportions of α, ω -dicarboxylic acids are mostly found in suberin. The phenolic components of suberin are derived from the phenylpropanoid pathway and mainly consist of hydroxycinnamates, commonly ferulic acid and coumaric acid, and monolignols (Vishwanath et al., 2015). The main differences between the chemical configuration of suberin and that of other cell wall biopolymers including lignin and cutin is the two distinct polymeric domains - poly(aliphatic) and poly(phenolic) (Bernards, 2002). These domains have separate and unique chemical composition, and both domains are present in the same cells. The poly(aliphatic) domain is known to be a glycerol-bridged, three-dimensional, polyester network of α, ω-dioic acids, ω-hydroxy acids, long-chain fatty acids, mid-chain-oxidized fatty acids, and esterified hydroxycinnamic acids. The suberin poly(phenolic) domain consists of a covalently cross-linked hydroxycinnamic acid/hydroxycinnamyl alcohol-derived matrix (Bernards, 2002).

1.14 Suberin distribution in plant tissue and functions

Suberin is usually deposited between the cell wall and plasma membrane of diverse tissues that interface with the environment (Bernards, 2002). A most prominent example of such a tissue is the suberized periderm (bark) of *Quercus suber,* commonly called the cork oak. According to Pereira et al. (2008), cork oak periderm is water impermeable and has good thermal insulating qualities. The usefulness of suberin in cork oak is wide ranging. It has found applications as an insulator and as a sealing material. Of course, in the wine industry, cork is used as a bottle stopper – due to its impermeability (Pereira et al., 2008).

Graca & Pereira (2000) state that tissues at the interface between the plant and the environment consists of up to more than 50% of suberin. Suberin is also accumulated together with its associated waxes in the cell walls of root epidermal, exodermal, and endodermal cells. Suberin is abundant in the skin (periderm) of the potato (*Solanum tuberosum)* tuber, where it serves to provide a physical barrier against loss of moisture and other environmental stresses as well as in preventing pathogen and insect invasion (Lulai & Freeman, 2001). Wounding of potato periderm leads to the induction and accumulation of new suberin deposition (Lukas Schreiber et al., 2005), and this serves as part of wound healing that helps to seal off the wound to prevent pathogen entrance, and functions to prevent water loss during post-harvest storage of potato tubers (Lulai, 2007). A study conducted using pathogen resistant and pathogen susceptible cultivars of potato showed that suberin forms a crucial barrier to prevent pathogen invasion of the potato tubers. It was shown that the polyaromatic domain is important for potato resistance against *Erwinia carotovora* subsp. carotovora (a bacterium), while the polyaliphatic domain is important for resistance against the fungus *Fusarium sambucinum* (Lulai & Corsini, 1998; Lulai & Freeman, 2001). The usefulness of suberin in reducing pathogen invasion was also shown by Ranathunge et al. (2008) using a soybean (*Glycine max*) line with a highly suberized cell wall. This soybean variety showed partial resistance to the oomycete *Phytophthora sojae* and displayed aliphatic suberin deposition up to 4 days earlier than a susceptible soybean line (Ranathunge et al., 2008). Arabidopsis also accumulates suberin in the endodermis of young roots, in the periderm of mature roots (Kreszies et al., 2018), and in the seed coat (Molina et al., 2008).

As a hydrophobic biopolymer, suberin deposition in specialized tissues both above and below the ground has important functions as a protective barrier to water diffusion as well as selective uptake of solutes from the soil environment (Baxter et al., 2009). As noted by Franke and Schreiber (2007) and Barberon, (2017), it is the aliphatic part of suberin that controls how it acts as a transport impediment, which plays crucial roles in regulating how plants take in water and nutrients (Barberon, 2017). Water and nutrients are transported through the root epidermis, cortex and endodermis before reaching the central vasculature, where the nutrients are transported upwards to the aerial parts of the plant. The presence of Casparian strips and suberin lamellae in the endodermis is crucial for successful nutrient uptake (Barberon, 2017). Passage of water and nutrients via the root are made possible by three pathways: the first is the apoplastic pathway, that allows free passage of materials by diffusion through the apoplastic space (the space outside the plasma membrane). Suberin deposition has no effect on apoplast transport because it is deposited on the inner surface of the primary wall and does not cover the spaces between cells. The second is the symplastic pathway, which is facilitated by plasmodesmatal connections between adjacent cells, and the third is the coupled transcellular pathway where polarized influx and efflux carriers transport solutes (Barberon, 2017) (Figure 1-6, Figure 1-7).

1.15 Biosynthesis, Transportation and Deposition of Suberin in Arabidopsis

Genetic studies in Arabidopsis have provided new insights into suberin biosynthesis. Vishwanath et al. (2015) described the enzymology of biosynthesis of suberin monomers. Enzymes identified for monomer biosynthesis are β-ketoacyl-CoA synthases (KCSs), fatty acyl reductases FARs, long-chain acyl-CoA synthetases, cytochrome P450

monooxygenases, fatty acid ω-hydroxylases (CYP86A1 and CYP86B1), acyl-CoA, glycerol 3-phosphate acyltransferases (GPATs), and phenolic acyltransferases (Vishwanath et al., 2015). Mutation in genes coding for suberin monomers biosynthetic enzymes results in mutants with various deficiencies in suberin content (Table 1-2).

1.15.1 Biosynthesis of suberin aliphatic monomers

The synthesis of suberin monomers begins with fatty acid synthesis, which occurs in the plastid and leads to the production of 16:0 and 18:1 fatty acyl-CoAs. The fatty acyl chains are exported to the cytosol where a series of modifications take place. The fatty acyl chains become adapted and elongated by suberin biosynthetic enzymes localized at the endoplasmic reticulum (ER) –called the fatty acid elongation (FAE) complex – via the sequential addition of C2 moieties from malonyl-CoA to the preformed C16 or C18 acyl groups, leading to the production of very long chain fatty acyls (≥C20, VLCFAs). Ketoacyl-CoA synthases (KCS), which are present as part of the endoplasmic reticulum-localized fatty acid elongation complex (FAE), are responsible for the first enzymatic step in the elongation of C16/C18 fatty acyl chains into VLCFAs (Millar & Kunst, 1997). The reducing enzyme DAISY/AtKCS2 was thought to be involved in suberin biosynthesis (Franke et al., 2009), because loss-of-function *daisy/kcs2* mutants have reductions in C22 and C24 VLCFA derivatives in root suberin (Franke et al., 2009).

In the cytosol, the activities of fatty acyl reductases (FARs) enzymes cause fatty acyl reduction to produce primary alcohols through an unreleased fatty aldehyde intermediate (Rowland & Domergue, 2012). There are eight members of the *FAR* gene family in Arabidopsis, out of which the gene expression patterns of *FAR1, FAR4* and *FAR5*

correspond with known sites of suberin deposition (Domergue et al., 2010). There is also fatty acyl oxidation by cytochrome P450 enzymes (CYPs), which produce ω-hydroxy fatty acids (ω-OHs) and α, ω -dicarboxylic acids (DCAs). The hydroxylation of the terminal methyl group of aliphatics (ω-position) is catalyzed by enzymes belonging to the CYP86 subfamily of cytochrome P450 monooxygenases (Molina, 2010). The fatty acids are hydroxylated to 16:0 and 18:1 ω-hydroxy fatty acids, and then further oxidized into α,ω-dicarboxylic acids by an ω-hydroxy fatty acid dehydrogenase (De Silva, 2019). Esterification of ω-OHs and DCAs to glycerol 3-phosphate (G3P) by glycerol 3-phosphate acyltransferases (GPATs) producing monoacylglycerols also occur at the ER membrane. How the building blocks of suberin are transported and polymerized intracellularly is yet to be fully understood. However, ATP-binding-cassette (ABC) transporters are thought to be involved in bringing suberin monomers from the point of production across the plasma membrane to the cell wall.

It is likely that polyester synthase(s) (PS) associated with the cell wall produce high molecular weight polyesters by extending monoacylglycerol with other suberin monomers. The products of the phenylpropanoid pathway – coumaric, caffeic, and ferulic acids – are linked to fatty alcohols by BAHD-type acyltransferases to produce alkyl hydroxycinnamates (AHCs) found in suberin-associated waxes. These enzymes include Aliphatic Suberin Feruloyl Transferase (ASFT) and Fatty Alcohol Caffeoyl-CoA Transferase (FACT). MYB-type transcription factors are important for controlling the specific developmental and stress-induced gene expression patterns of the suberin biosynthetic genes (Vishwanath et al., 2015) (Figure 1-8).

1.15.2 Transportation and deposition of suberin monomers

Once the monomer or partially formed oligomers are synthesized, there is need for their transport from the ER to and across the plasma membrane, prior to polymerization in the apoplast and deposition to form the suberin lamellae (Vishwanath et al., 2015). Despite knowledge of the biosynthetic pathway of suberin aliphatics monomers, how they are transported and polymerized is not yet clear. However, Yadav et al. (2014), identified three members of ATP-Binding Cassette G transporters subfamily, ABCG2, ABCG6 and ABCG20, as being involved in the conveyance of suberin monomers from the ER to the apoplast for suberin lamellae deposition (Mahmood et al., 2019). Lee & Suh (2018) also identified a member of glycosylphosphatidylinositol (GPI)-anchored lipid transfer proteins (LTPG15) that may be involved in suberin transport.

It was revealed that the triple mutant*, abcg2 abcg6 abcg20,* has an imperfect suberin barrier formation in roots and seed coats. Compared to the wild type, the suberin lamellae structure in this mutant is distorted and its seeds are highly porous to tetrazolium salts. In addition, the total suberin polyester monomer present in the seed coat of the triple mutant is less than that of wild-type seeds (Yadav et al., 2014). It was also discovered that roots in the triple mutant accumulate higher amounts of suberin monomers than the wild type and contained higher levels of transcripts of the suberin biosynthetic enzymes GPAT5, CYP86A1 and CYP86B1. These increased transcript levels may be responsible for the observed increases in suberin monomer content (Yadav et al., 2014). That suberin accumulated in the mutant roots, but with a distorted deposition pattern, may be because of defects in monomer transportation.

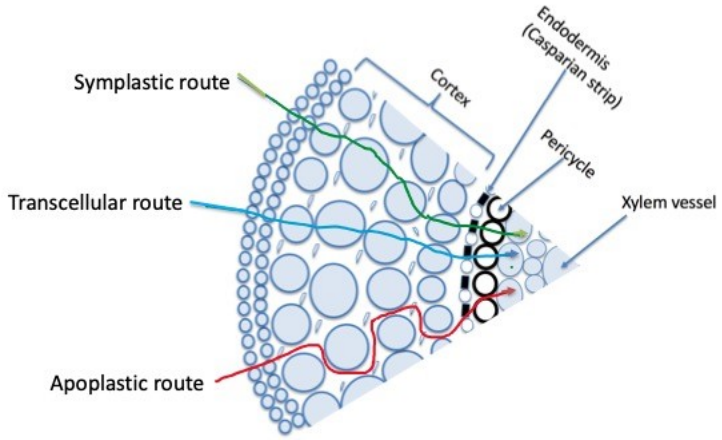


Figure 1-6. Schematic representation of three different pathways of water and solutes movement in roots. (Figure modified from Kim et al. (2018)).

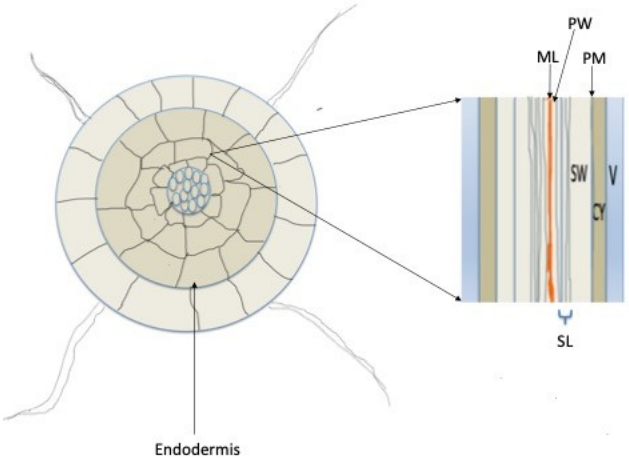
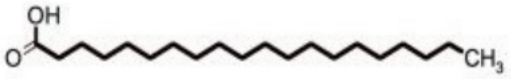
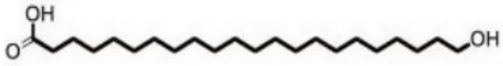
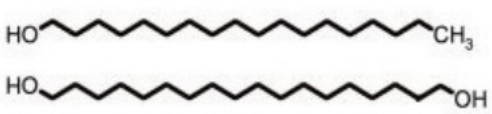
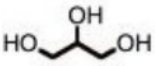
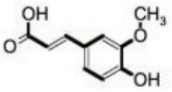


Figure 1-7. Suberin accumulation in the endodermis of a root.

Suberin lamellae (SL) deposited at the inner face of the primary cell walls. Secondary cell wall (SW) between the suberin lamellae and the plasma membrane (PM). Cy - cytoplasm; V – vacuole, ML – middle lamellae, PW – primary cell wall. See text for details. (Adapted from Beisson et al. (2012)).

Table 1-1. Structural formulae of the main suberin monomers

(Adapted from Graça (2015)).

Monomers	Chemical structure
Unsubstituted fatty acids (C 16 - C 26)	
ω-hydroxy fatty acids (C 16 - C 26)	
α, ω-dicarboxylic acids (C 16 - C 26)	
Fatty alcohols and diols (C 18 - C 22)	
Glycerol	
Ferulate	

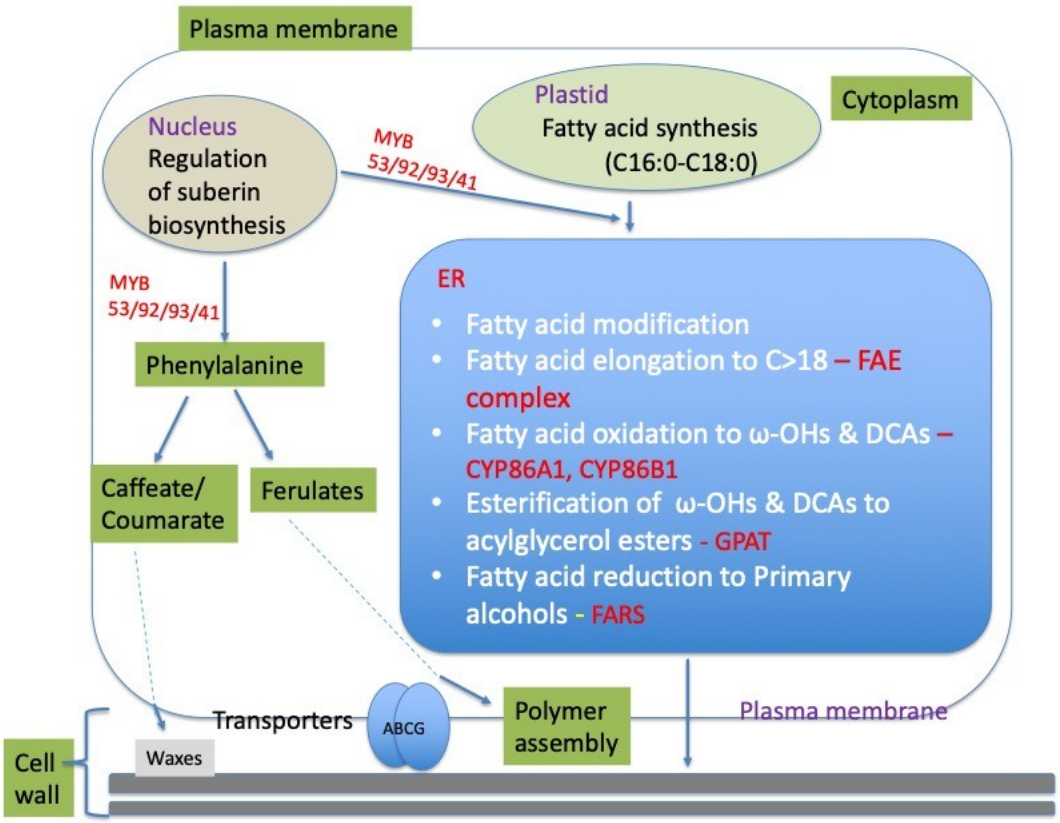


Figure 1-8. Schematic diagram summarizing major steps in suberin biosynthesis.

Suberin biosynthesis begins in the plastid with fatty acid synthesis to produce mainly 16:0 and 18 carbon acyl chains. At the endoplasmic reticulum**,** fatty acyl modifications occur including: (a) elongation occurs via the endoplasmic reticulum (ER)-associated fatty acid elongation (FAE) complex, producing very long chain fatty acyl chains, (b) Acyl reduction by fatty acyl reductases (FARs) to give rise to primary alcohols, (c) fatty acyl oxidation by cytochrome P450 enzymes (CYPs) produces ω-hydroxy fatty acids (ω-OHs) and α,ω - dicarboxylic acids (DCAs), (d) esterification of ω-OHs and DCAs to produce glycerol-3phosphate (G3P) by glycerol 3-phosphate acyltransferases (GPATs) to produce monoacylglycerols. Transport and polymerization mechanisms of suberin are unclear although suberin monomers are believed to be transported across the plasma membrane via ATP-Binding Cassette (ABC) transporters. Coumaric, caffeic, and ferulic acids are high molecular weight polyesters produced by the phenylpropanoid pathway and are linked to fatty alcohols to produce alkyl hydroxycinnamates (AHCs) of suberin-associated waxes. MYB-type transcription factors are believed to control the genes expressed during suberin biosynthesis in response to stress or development. (Figure adapted from Vishwanath et al. (2015)).

1.16 Regulation of biosynthesis, transport and polymerization of suberin monomers

How deposition of suberin is regulated is also unclear. It is thought that suberin deposition is controlled at the transcription level, and this is supported by gene expression studies of suberin biosynthetic genes (Ranathunge & Schreiber, 2011; Vishwanath et al., 2015). Genes of the MYB (Myeloblastosis) family of transcription factors (TF) have been identified that positively regulate suberin biosynthesis in different plant species. MYBs are a group of transcription factors that share a common DNA-binding domain (Cominelli et al., 2008) that exist in all eukaryotes. The most abundant MYB protein group in plants is the R2R3 subfamily of which members have two MYB repeats in their DNA-binding domains and are involved in the regulation of many plant specific processes including responses to abiotic and biotic stresses (Cominelli et al., 2008).

Cominelli et al. (2008) report that *AtMYB41* gene expression is induced in response to abiotic stress in wild-type plants, and overexpression of *AtMYB41* under the control of the CaMV 35S promoter produced a cuticle mutant phenotype, suggesting a possible role for AtMYB41 in the regulation of cuticle biosynthesis. Kosma et al. (2014) also revealed that when the transcription factor AtMYB41 was overexpressed, it leads to the activation of all steps necessary for aliphatic suberin synthesis and deposition of cell wall-associated suberin-like lamellae in both Arabidopsis and *Nicotiana benthamiana* leaves. A further role of stress-induced suberin biosynthesis was assigned to AtMYB41 because its expression and suberin deposition in root endodermal cells were induced by ABA and drought stresses.

Furthermore, the biosynthesis of suberin in Arabidopsis seed coats was positively regulated by two paralogous *MYB* genes, encoding MYB107 and MYB9, which directly

bind and activate the expression of suberin biosynthesis genes (Gou et al., 2017). Loss-of-function *myb107* mutants display significant (50%-60%) decreases in seed coat suberin, with higher permeability to tetrazolium salts than wild-type seeds (Gou et al., 2017). Capote et al. (2018) showed that QsMYB1, another member of MYB family in cork oak tree, positively regulates the expression of genes involved in suberin biosynthesis where it is directly involved in cork development. However, a member of the NAC transcription factor family, StNAC103, negatively regulates the accumulation of suberin polyester and associated waxes in potato tuber skin (Verdaguer et al., 2016).

Table 1-2. Nature of BOP, TGA, and suberin mutants used

Genotype / allele	Locus and T-DNA insertion line	Reference
Loss-of-function mutants		
bop1-3	SALK_012994	(Hepworth et al., 2005)
bop2-1	SALK_075879	(Hepworth et al., 2005)
tga1-1	SALK_028212	(Kesarwani et al., 2007)
tga4-1	SALK_127923	(Kesarwani et al., 2007)
bop1-3 bop2-1 tga1-1 tga4-1		(Wang et al., 2019)
cyp86b1-1	SM_37066 AT5G41040	(Compagnon et al., 2009)
cyp86b1-2	SALK_130265 AT5G41040	(Compagnon et al., 2009)
cyp86a1-1	At5g58860)	Höfer et al., 2008
far1-2	SALK_149469 AT5G22500	(Domergue et al., 2010)
far4-1	SALK_000229 AT3G44540	(Domergue et al., 2010)
far5-1	SALK_152963 AT3G44550	(Domergue et al., 2010)
abcg2-1	GABI_036B02 AT2G37360	(Yadav et al., 2014)
abcg6-1	At5G13580	(Yadav et al., 2014)
abcg20-1	At3G53510; SALK_011548C	(Yadav et al., 2014)
myb92-1	AT5G10280 SM_3_41690	Murmu et al., (unpublished)
myb93-1	AT1G34670 SALK_131752	Murmu et al., (unpublished)
Gain-of-function mutants		
bop1-6D	Activation tagged line, 4X CaMV 35S enhancer in *BOP1* promoter	(Norberg et al., 2005)
MYB41-OE	CaMV 35S:: *AtMYB41* promoter-driven	(Cominelli et al., 2008; Kosma et al., 2014)

1.17 Objectives

My research investigates a form of physical/chemical barrier and a form of induced immunity in plant using Arabidopsis model plant. There may be interactions in these general classes of plant defense against pathogens. The research is divided into two sections. The first objective focuses on investigating the role of BOP1/2 in plant defense. My second objective focuses on investigating Arabidopsis wild type and suberin mutants with altered amounts of suberin for disease susceptibility and/or resistance to fungal and bacterial pathogens. An associated goal of my thesis was the development of plant pathology assays to use in examining host and pathogen phenotype effects (pathosystem model for studying plant-pathogen interactions) for the study of resistance mechanisms. This was carried out by direct pathogen testing to study the hypotheses that:

1: BLADE-ON-PETIOLE genes have a broad-spectrum role in plant defense. Predictions: Plants that overexpress BOP1 or BOP2 will have increased resistance to both fungal and bacterial pathogens. Conversely, plants that are defective in BOP1/2 activity are predicted to be less resistant than wild type to infection with fungal and bacterial pathogens.

2: BLADE-ON-PETIOLE genes are required in pattern-triggered innate immunity and function in the same pathway as clade I TGAs. Predictions: Plants that overexpress BOP1 or BOP2 will exhibit increased hallmarks of a PTI defense response. Conversely, plants that are defective in BOP1/2 activity are predicted to show reduced PTI defense responses.

3. Cell wall suberization acts as barrier to the invasion of plant root fungal pathogens Prediction: mutant plants deficient in root suberin deposition will have increased susceptibility to pathogens, compared to the wild type.

4. Cell wall suberization in root endoderm and or periderm acts as barrier to the loss of water via the roots of *A. thaliana*. Prediction: mutant plants deficient in root suberin deposition will have increased water loss via the roots in comparison to the wild type

5. Ectopic suberin expression in Arabidopsis leaves acts as barrier to the invasion of leaf bacterial and fungal pathogens. Prediction: mutant plants ectopically expressing suberin will have increased resistance to leaf bacterial and fungal pathogens, compared to the wild type.

6. Ectopic suberin expression in Arabidopsis leaves acts as barrier to loss of water through the leaf surfaces. Prediction: mutant plants ectopically expressing suberin will have reduced water loss through the surface, compared to the wild type.

CHAPTER 2: Investigating the role of *BLADE-ON-PETIOLE* genes of Arabidopsis in defense against bacterial and fungal pathogens

2.1 Abstract

Plants employ both innate and induced immunity to defend against pathogens. Arabidopsis BLADE-ON-PETIOLE 1 and 2 (BOP1/2) form a subclade of BTB-ankyrin transcription co-factors within the NONEXPRESSER OF PATHOGENESIS-RELATED GENES 1 (NPR1) family of defense regulators. BOP1/2 were originally found to control plant development but their placement within a family that confers systemic acquired resistance (SAR) prompted my investigation of a role in plant defense. I therefore used pathogen assays to test a requirement for BOP1/2 plant defense leading to two key findings. First, BOP1/2 promote resistance to diverse bacterial and fungal pathogens. Second, BOP1/2 contribute to pattern-triggered immunity. My work demonstrates broad roles for BOP1/2 in plant immunity. Future work will address the mechanisms.

2.2 Introduction

2.2.1 *BLADE-ON-PETIOLE* genes of Arabidopsis regulate plant development

BOP1 and BOP2 belong to a family of transcription co-factors containing a BTB/POZ (Broad-complex, Tramtrack, and Bric-a-brac/POX virus and zinc finger) domain and ankyrin repeats (Khan et al., 2014). In mosses and higher plants, BTB-ankyrin proteins form two phylogenetic subclades (Backer et al., 2019). NPR1 and its three closest homologs (NPR2, NPR3, and NPR4) regulate salicylic acid-mediated systemic acquired resistance (Backer et al., 2019; Canet et al., 2010; Cao et al., 1997; Ding et al., 2018; Fu et al., 2012). BOP1 and BOP2 form a second subclade that functions in plant development. These two BTB-ankyrin factors regulate plant architecture, mainly through their activity at

organ boundaries. Boundaries are regions of low growth that separate emerging organs from the meristem. These regions become attachment points where organs join the plant body. BOP1/2 regulate growth in boundaries to control the shape and complexity of leaves and axillary shoots. BOP1/2 also regulate the formation of abscission zones (Hepworth et al., 2005; Khan et al., 2014). Major boundary-related defects in *bop1 bop2* mutants include organ fusions, elongated leafy petioles, abnormal floral patterning, and defects in floral organ abscission (Hepworth et al., 2005; Norberg et al., 2005). Constitutive expression of BOP1 or BOP2 results in a dwarf phenotype (Norberg et al., 2005). As transcription co-factors, BOP1 and BOP2 have an activation domain but interact with TGACG-motif binding (TGA) basic leucine zipper (bZIP) proteins for DNA binding. Arabidopsis has ten TGA factors organized into five subclades (Gatz, 2013). BOP1/2 interact with clade V TGA8/PAN to specifically regulate flower development (Hepworth et al., 2005; Xu et al., 2010). More broadly, BOP1/2 interact with clade I TGA1 and TGA4 to regulate boundary functions (Wang et al., 2020). The developmental significance of BOP1/2 interactions with clade III TGA3 and TGA7 transcription factors is currently under investigation.

TGA1 and *TGA4* expression is concentrated at organ boundaries but functional redundancy means that boundary defects are not easy to detect in *tga1 tga4* double mutants. Gain of function studies using plants that overexpress BOP1 or BOP2 revealed such a role. For example, the dwarf phenotype of *35S:BOP2* plants was rescued by crossing to a *tga1 tga4* mutant, showing that BOP2 requires the activity of clade I TGAs to exert changes in morphology. Further experiments revealed that *TGA1* and *TGA4* are under common regulation with other boundary genes and function in the same genetic pathway as BOP1/2 required for meristem integrity, flower development, and inflorescence architecture.

Mechanistically, it was shown that TGA1 and TGA4 form heterodimers with BOP1 and BOP2, contributing to the activation of homeobox gene *ARABIDOPSIS THALIANA HOMEOBOX GENE1*, which is needed for boundary establishment (Wang et al., 2019).

Interestingly, clade I TGAs were previously described as having roles in plant immunity. Loss-of-function *tga1 tga4* mutants are deficient in basal resistance to bacterial pathogen infections, showing a reduction in apoplastic defenses and impaired SA biosynthesis (Sun et al., 2018; Wang & Fobert, 2013). Clade I TGAs also function in SA mediated defense, subject to redox modifications that allow their interaction with NPR1 for the induction of SA responsive gene expression (Després et al., 2003). Given the dual role of clade I TGAs in development and defense, BOP1/2 may likewise function in both processes.

2.2.2 Evidence for a role in plant defense

An initial characterization of the *bop1 bop2* mutant showed no change in resistance to a bacterial biotrophic pathogen (Hepworth et al., 2005). It was later reported that *bop1 bop2* mutants lack a priming response to methyl jasmonate (MeJA) suggesting a possible role in JA-mediated defense responses (Canet et al., 2012). A microarray study of *BOP1*-overexpressing plants showing the increased expression of genes in JA, SA, and ET-related signaling pathways provided further evidence of a defense role (Khan et al., 2012). Gene Ontology (GO) enrichment analysis was carried out to further test this link. Approximately one third of up-regulated genes (1106 out of 2928, 38%) were classified as “stimuli-related”. Further classification showed that 291 of 700 genes sorted to plant biotic defense pathways. The highest proportion of genes was associated with a PTI defense response

(55.7%) followed by JA/ET-mediated defense pathways (49.8%) and SA-associated defense pathways (33.7%). Validation by RT-qPCR confirmed that transcripts for many PTI-associated genes including *TGA1* and *TGA4* were up-regulated in *BOP1*-over expressing plants (Wang et al., 2020). These findings promoted my direct testing of disease resistance in BOP1/2 loss and gain of function mutants.

2.2.3 Rationale for the study of BLADE-ON-PETIOLE genes as defense genes

Multiple lines of evidence suggest that BOP1/2 may have roles not only in development, but also in plant defense against pathogens based on the following:

1. BOP1/2 are homologous to NPR1, and NPR1 has been shown to play roles in plant defense against pathogens (Backer et al., 2019; Khan et al., 2014).
2. *BOP1/2* genes are essential for resistance induced by methyl jasmonate (MeJA) in Arabidopsis and modify plant responses to SA (Canet et al., 2012; Castelló et al., 2018).
3. Microarray data suggested a strong link between *BOP1* gene activity and defense pathways (Wang et al., 2020).
4. Clade I TGA bZIP transcription factors that promote SA biosynthesis and contribute primarily to innate immunity, are required by BOP to regulate plant development (Wang et al., 2019).

2.2.4 Goals and Hypotheses

Here, I examined the role of Arabidopsis *BOP1/2* genes in plant defense based on the evidence above.

Hypothesis 1: *BLADE-ON-PETIOLE* genes have a broad-spectrum role in plant defense. Predictions: Plants that overexpress BOP1 or BOP2 will have increased resistance to both fungal and bacterial pathogens. Conversely, plants that are defective in BOP1/2 activity are predicted to be less resistant than wild type to infection with fungal and bacterial pathogens.

Hypothesis 2: *BLADE-ON-PETIOLE* genes are required in pattern-triggered innate immunity and function in the same pathway as clade I TGAs. Predictions: Plants that overexpress BOP1 or BOP2 will exhibit increased hallmarks of a PTI defense response. Conversely, plants that are defective in BOP1/2 activity are predicted to show reduced PTI defense responses.

2.3 Materials and Methods

2.3.1 Plant material and growth conditions

Arabidopsis thaliana Columbia-0 (Col-0) ecotype was used as the wild type (WT) in this study. Mutant alleles of *BOP1* and *BOP2* used in this study were the *bop1-6D* gain-of-function mutant (*BOP1-oe*) (Norberg et al., 2005), loss-of-function *bop1-3 bop2-1* double mutant (Hepworth et al., 2005), *tga1-1 tga4-1* double mutant (Kesarwani et al., 2007), and *bop1-3 bop2-1 tga1-1 tga4-1* quadruple mutant (Wang et al., 2019).

Seeds were surface sterilized by rinsing in 100% ethanol and then transferred to a freshly made solution of 5% sodium hypochlorite (bleach) and 0.5% (w/v) sodium dodecyl sulphate (SDS). After one minute of incubation, the seeds were rinsed four times in sterile distilled water and sown on agar plates containing minimal media (Haughn & Somerville, 1986) for plant propagation, or directly in trays of steam-sterilized soil (ProMix BX, Premier Horticulture, Riviere-du-loup, QC) supplemented with a 1 g/L^{-1} solution of 20-20-20 plant fertilizer (Plant-Prod Inc., Brampton, ON). Plated seeds were wrapped in foil and incubated at 4ºC in the dark for 2-3 days to break dormancy. Steam-sterilized soil seeds were covered with black trays and kept in the cold room for 2-3 days to break dormancy. Following cold treatment, plates and trays were moved into growth chambers for germination. Plates for propagation were moved to growth chambers under continuous light (~115 µmol m^{-2} sec^{-1}) at 21ºC. After germination, seedlings for propagation were transplanted from plates to trays of steam-sterilized soil with fertilizer and grown to maturity in growth chambers under continuous light (~115 µmol m^{-2} sec^{-1}) at 21ºC. Seedlings for experiments were transplanted to trays of steam-sterilized soil with fertilizer and grown under short days (8 h light/16 h dark) or long days (16 h light/8 h dark) with a light intensity of ~115 µmol m^{-2} sec^{-1} at 21-22ºC.

2.3.2 Pathogen cultivation and maintenance

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000) containing pVSP61 (empty vector) was kindly provided by Gopal Subramaniam (Agriculture and Agri-Food Canada). The strain was maintained as described by Ritter & Dangl (1996). Bacteria were

grown, inoculated, and assayed for growth on the plant as described by Tornero & Dangl (2001).

Botrytis cinerea strain RW1A6P (Micalizzi et al., 2017) was isolated from rotting wood in Ottawa, Ontario by Emma Micalizzi. The pathogen was cultured and maintained on potato dextrose agar (PDA). *Botrytis cinerea* (RW1A6P) was inoculated and assayed for infection on the plant according to (Murmu et al., 2014).

2.3.3 Pathogen assays

2.3.3.1 *Pseudomonas syringae* assay

Plants were assayed for resistance against *Pseudomonas syringae* pathovar (pv) *tomato* DC3000 (*Pst* DC3000) or a *hrcC*$^-$ mutant (*Pst hrcC*$^-$) as described (Shearer et al., 2012; Wang & Fobert, 2013). In brief, bacteria were grown at 28°C on King's B agar medium containing rifampicin (100 mg l^{-1}). Overnight cultures were resuspended in infiltration medium (10 mM $MgCl_2$). Leaves of four-week-old plants grown under short days were syringe-infiltrated with bacteria at 10^5 cell ml^{-1} (OD600 = 0.0002) and returned to the growth chamber. To determine the bacterial growth in Arabidopsis leaves, the internal bacterial population was measured at 0, 1, 2 and 3 days post inoculation (dpi) using three replicates per genotype. Each replicate consisted of 4 leaf discs (1 cm^2) from the infected leaves of four different plants. Samples were homogenized in sterile water and bacterial titers were determined by plating serial dilutions of the homogenized samples on King's B agar medium containing rifampicin (100 mg l^{-1}) as described (Yao et al., 2013). Two days after plating, the average number of the bacterial cfu per cm^2 was calculated and

log-transformed data were analyzed. The data shown are representative of three independent experiments.

2.3.3.2 *Botrytis cinerea* assay

Plants were assayed for resistance against *Botrytis cinerea* infection as described by (Murmu et al. (2014). Spores of *B. cinerea* strain RW1A6P (Micalizzi et al., 2017) were harvested from potato dextrose agar (PDA) plates into sterile water. The spore suspension was filtered through four layers of sterile cheesecloth and washed twice in sterile water. The spores were resuspended in potato dextrose broth (PDB) and diluted to a concentration of 5×10^5 ml^{-1} for pathogenicity tests. Detached leaves from four-week-old plants grown under long days at 22ºC were placed separately on three layers of moist sterile Whatman filter paper (Fisher Scientific, ON, Canada) inside a plastic petri dish. The center of each leaf was inoculated with 6 µL of the spore suspension. PDB was used to dilute the spores and not water, so the 6 µL drop of spore suspension can stay at the centre of the leaf. The plates were sealed with surgical tape to maintain humidity and incubated in the dark for the first 12 hours and then transferred to a growth chamber set at 22°C under long days and monitored for four days. The diseased leaves were photographed at daily intervals to record lesion spread. Lesion diameters were measured from the photographs using ImageJ software (http://www.nih.gov).

2.3.4 Oxidative burst assay

ROS production from leaf tissue was measured as described (Brauer et al., 2016). Briefly, duplicate 4-mm leaf disks were collected from the fully expanded leaves of four-week-old plants (avoiding the mid rib), using 8 plants per genotype. Disks were floated on deionized water overnight with the adaxial side up in a 96-well plate. The following day, the water was replaced with 100 µl of detection buffer containing [17 µl ml^{-1} luminol (Sigma-Aldrich, St. Louis, MO, USA), 20 µl ml^{-1} horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) and 1 µM flg22 peptide (PhytoTechnology Laboratories, Lenexa, KS, USA)]. Luminescence was measured for 45 min after buffer application using an Infinite® 200PRO microplate reader (Tecan Group Instruments, Männedorf, Switzerland). Relative light units were calculated for each time point within an experiment. The experiment was performed three times with similar results.

2.3.5 Callose staining

Callose deposition was monitored according to (Schenk & Schikora, 2015). In brief, leaves of four-week-plants were infiltrated with *Pseudomonas syringae* DC3000 *hrcC*$^{-}$ at a dose of 10^5 cfu ml^{-1} or 10 mM $MgCl_2$ (mock). Alternatively, leaves were infiltrated with 1 µM flg22 peptide (AnaSpec Catalog # AS-62633) or sterile distilled water (mock). After 12 hours of treatment, whole leaves were fixed and cleared overnight in a 1:3 solution of acetic acid/ethanol (v/v) until the leaves were transparent. The next day, samples were stained in 0.01% aniline blue in 150 mM K_2HPO_4 for 12 hours in in the dark. The next day, samples were mounted in 50% glycerol and imaged under UV light using a compound microscope (Axio Imager M2, Carl Zeiss, Germany or Axio Imager, Carl Zeiss Canada,

North York, ON). For quantification of callose deposits, three leaves were collected from three plants per genotype. Three areas on each leaf were photographed for manual counting of callose deposits (Schenk & Schikora, 2015).

2.3.6 Protein extraction and western blot analysis

Leaves of four-week-old plants grown under short days were syringe-infiltrated with *Pst* D3000 or *Pst hrcC*$^{-}$ bacteria at dose of 10^8 cfu ml^{-1}. Leaf tissues from three plants were collected and pooled as one sample for intercellular washing fluids (IWFs) and total protein extraction. IWF and total protein were collected at 0 and 2 dpi, separated on 16% SDS-polyacrylamide gels, and blotted with a PR1 antibody as described (Wang & Fobert, 2013). Briefly, infected leaves were harvested on stipulated days and infiltrated under vacuum in IWF buffer containing [50 mM sodium phosphate buffer pH 7.5, 600 mM NaCl, 0.01% Tween-20, 0.1% β-mercaptoethanol] at 4°C in square petri dishes. IWF was collected from equal amounts of tissue by centrifuging the infiltrated leaf samples, which were packed in a syringe, for 5 min at 1500g. Total protein was also extracted using protein extraction buffer as described by (Wang & Fobert, 2013). In brief, infected leaves were harvested and frozen immediately in liquid nitrogen, ground to a powder, and scraped into extraction buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 5 mM DTT) including phosphatase inhibitor (Sigma-Aldrich, part number 4906845001) and protease inhibitor cocktail (Sigma-Aldrich, part number P9599).

The insoluble debris was pelleted by centrifugation at 20,000 × g for 10 min at 4°C. Protein concentration was determined using the Bio-Rad protein assay (http://www.bio-rad.com). 50 μg aliquots of IWF protein were run on 12% Tricine-SDS-PAGE gels (mini

protein, Bio-Rad) and transferred to a nitrocellulose membrane. Western blots were carried out by standard methods and probed with antibodies specific to the PR1 protein (Agrisera, part number AS10 687) and anti-rabbit horseradish peroxidase (HRP) antibodies (Abcam, part number ab97051). The blots were developed with an enhanced chemiluminescence detection system, according to the manufacturer's instructions (HRP/Luminol Chemiluminescent Detection Kit and ThermoFisher SuperSignal™ West Pico PLUS Chemiluminescent Substrate, catalog number 34580). The same gels were stained with Ponceau stain as a loading control. The Arabidopsis PR1 protein has a predicted molecular weight of about 17 kilodaltons (kDa).

2.4 Results

2.4.1 *BLADE-ON-PETIOLE* genes confer resistance to diverse pathogens

Arabidopsis *BOP1* and *BOP2* genes are strongly expressed at organ boundaries where growth in restricted. Consequently, the overexpression of these genes causes dwarfing. Microarray transcriptome analysis of *BOP1*-overexpressing (*BOP1-oe*) plants linked this phenotype with an increase in expression of defense-related genes from different pathways (Khan et al., 2015; Wang et al., 2020). To test if this increase in defense-related gene expression confers resistance to pathogens, *BOP1-oe* plants were challenged with the hemi-biotrophic bacterium *Pseudomonas syringae* pv. *tomato* (Pst) DC3000, which elicits a strong SA-dependent defense response (Kim, et al., 2008) and with *Botrytis cinerea*, a necrotrophic fungal pathogen that elicits a strongly JA/ET-dependent defense response (Windram et al., 2012).

2.4.1.1 Response of *BOP* mutants to *Pseudomonas syringae* infection

Disease resistance was first tested using *Pseudomonas syringae*, which activates SA-dependent defense mechanisms (Carviel et al., 2014; Xin et al., 2018). Four-week-old leaves of wild-type, *BOP1-oe* and *bop1 bop2* plants grown under short days were syringe-infiltrated with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) at a dose of OD_{600} = 0.0002 (equivalent to 10^5 colony-forming units per ml). Bacteria occurring within the plant tissue was enumerated at 0, 1, 2, and 3 days following infiltration. At all timepoints following infiltration, *BOP1-oe* leaves showed a substantial resistance to *Pst* DC3000, with leaves supporting significantly lower bacterial counts compared to wild type control leaves. By contrast, the bacteria grew to significantly higher titres in *bop1 bop2* leaves, indicating a greater susceptibility compared to the wild type (Figure 2-1).

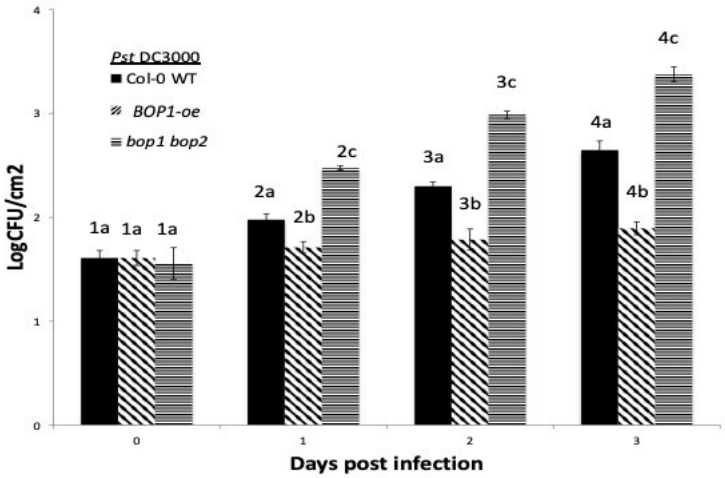


Figure 2-1. Growth of hemi-biotrophic bacterial pathogen *Pst* DC3000 in wild type, *BOP1-oe,* and *bop1 bop2* plants.

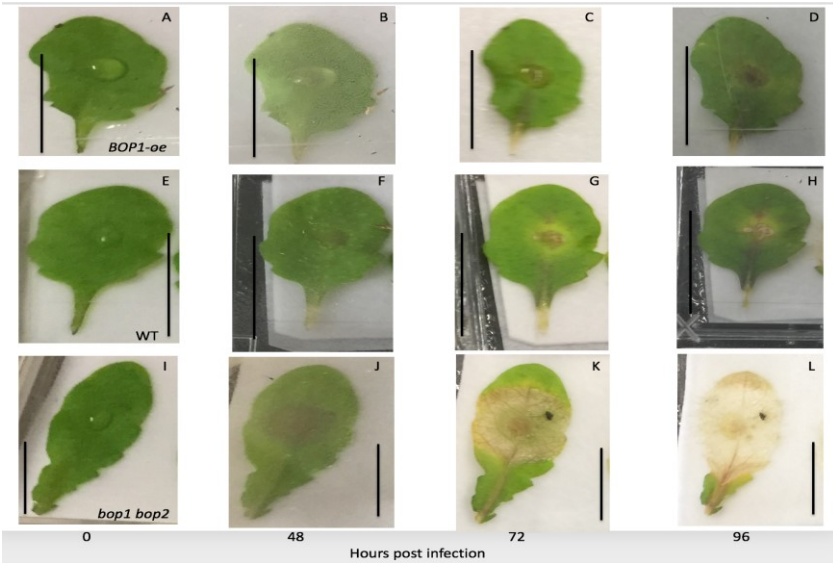
Leaves of four-week-old plants grown under short days were syringe-infiltrated with bacteria at 10^5 colony forming units (cfu) ml^{-1}. Log-transformed data are means ± SD of three replicates, each containing four leaf disks from separate plants. Bars designated by a, b or c indicate significance differences of bacteria recovered on a given day, based on 1-way ANOVA with post hoc Tukey's HSD test ($p<0.05$).

2.4.1.2 Response of *BOP* mutants to *Botrytis cinerea* infection

To further test the involvement of *BOP* genes in disease resistance, plants were challenged with *Botrytis cinerea*, a necrotrophic fungal pathogen that elicits a strong JA/ET-dependent defense response (Windram et al., 2012). The *B. cinerea* strain RW1A6P (Micalizzi et al., 2017) was used in pathogenicity trials. Fungal growth and proliferation was measured in wild-type, *BOP1-oe* and *bop1 bop2* plants. Detached leaves of four-week-old plants grown under long days were infected at the centre with 6 µl of *Botrytis cinerea* spores (5×10^5 spores per ml) and photographed at 0, 2, 3 and 4 days after inoculation to measure lesion size. Results showed that *BOP1-oe* plants were significantly more resistant to *B. cinerea* than wild type plants. Four days after inoculation, the lesion diameter on *BOP1-oe* leaves (2.3 ± 0.03 mm) was significantly smaller compared to wild-type leaves (3.8 ± 0.05 mm) indicating resistance. In contrast, the lesion on *bop1 bop2* leaves developed rapidly and reached a diameter (11.2 ± 0.7 mm) that was ~3-fold larger than wild type, indicating susceptibility (Figure 2-2).

In summary, pathogen trials showed that constitutive expression of *BOP1* confers resistance to bacterial and fungal pathogens. By contrast, *bop1 bop2* mutants were more susceptible compared to wild-type plants—particularly to *B. cinerea* infection where defense relies on JA/ET-mediated signaling. These collective results support a broad-spectrum role for Arabidopsis BOP1 and BOP2 in disease resistance.

A



B

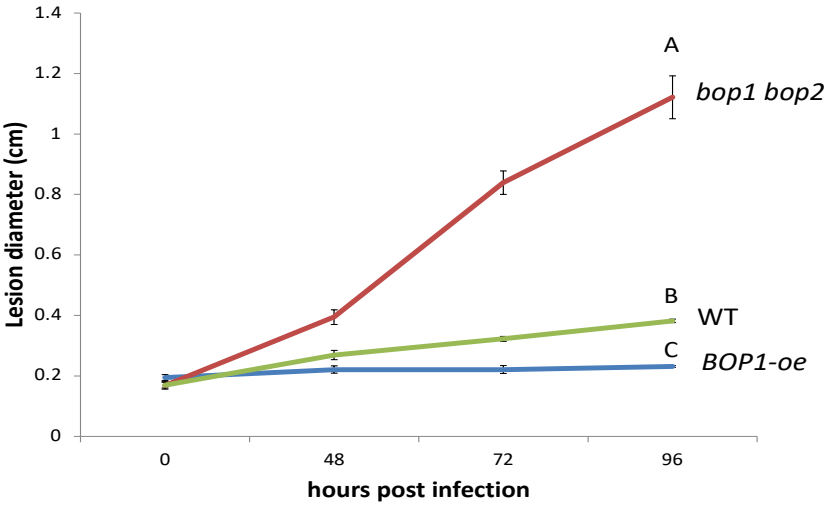


Figure 2-2. Growth of necrotrophic fungal pathogen *B. cinerea* in wild type, *BOP1-oe,* and *bop1 bop2* plants.

A) Photographs showing lesions produced by *Botrytis cinerea* at 0, 48, 72 & 96 hours post inoculation (*hpi) (A-D) BOP1-oe; (E-H)* Col-0 WT; *(I-L) bop1 bop2.* Detached leaves of four-week-old plants grown under long days were infected with 6 µl of *B. cinerea* spores (5 x 10^5 spores ml/1) and photographed as indicated for measurement of lesion size using 15 leaves per genotype. B) Graph of lesion diameter measurement shows significant differences among the three mutants. *BOP1-oe* is most resistant to pathogen, whereas *bop1 bop2* is highly susceptible compared to WT. Data are means ± SD from 10 infected leaves using five plants per genotype. Different letters indicate significant differences ($p \leq 0.01$, one-way ANOVA, HSD post hoc, $\alpha=0.05$) in lesion measurements at 96 hours post infection.

2.4.2 BLADE-ON-PETIOLE genes are required in PTI and potentially function in the same pathway as clade I TGAs.

GO analysis of up-regulated genes in *BOP1-oe* plants resolved by microarray identified a large fraction of genes associated with PTI where clade I TGAs are active (Wang et al., 2020). Earlier studies by Wang & Fobert (2013) showed that the *tga1 tga4* mutant is compromised in defense against *Pst hrcC*$^{-}$ and many hallmarks of PTI including the oxidative burst, callose deposition, and PR1 protein secretion show a reduced response. The *hrcC*$^{-}$ mutant of *Pst* DC3000 (*Pst hrcC*$^{-}$) is specifically used to assay PTI because this mutant lacks a functional apparatus for the delivery of ETI-triggering effectors into the plant cell (Hauck et al., 2003). Treatment of plants with a conserved 22 amino acid fragment of bacterial flagellin (flg22) is also a specific trigger for PTI (Zipfel et al., 2004).

2.4.2.1 Response of *BOP* mutants to *Pst hrcC*$^{-}$ infection

To first test effects on PTI, the leaves of wild-type and *BOP1-oe*, *bop1 bop2, tga1 tga4,* and *bop1 bop2 tga1 tga4* mutants were infiltrated with *Pst hrcC*$^{-}$ bacteria. Four days after infiltration, the leaves of *BOP1-oe* harboured significantly fewer bacteria compared to wild-type, indicating resistance. In contrast, the bacterial titres in *bop1 bop2, tga1 tga4* and *bop1 bop2 tga1 tga4* leaves were significantly higher than in wild-type control plants (Figure 2- 4). These data showing resistance for *BOP1-oe* plants versus similarly increased bacterial titres in *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* mutants support the idea that BOP1/2 and clade I TGA genes might function in the same genetic pathway to promote PTI.

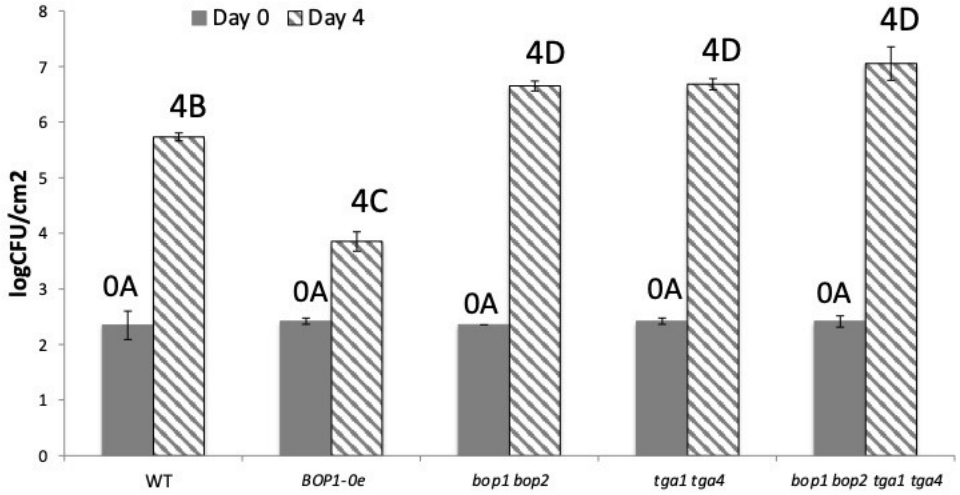


Figure 2-3. Growth of *Pst hrcC*⁻ mutant of *Pseudomonas syringae* pv. *tomato* DC3000 in wild type, *BOP1-oe, bop1 bop2, tga1 tga4* and *bop1 bop2 tga1 tga4.*

Log-transformed data are means ± SE of three replicates, each containing four leaf disks from separate plants. Different letters indicate significant differences between wild type and mutants ($p<0.05$, one-way ANOVA with post hoc Tukey's HSD test) on day 0 (no differences) and at day 4.

2.4.2.2 Oxidative burst assay

A transient apoplastic burst of ROS is an early response of plant cells to the activation of pattern recognition receptors involved in PTI. In Arabidopsis, ROS levels begin to increase within 2-3 minutes and reach a peak around 6-10 minutes after pathogen perception (Daudi et al., 2012; Torres et al., 2006). This extracellular oxidative burst is due to action of the RBOH family of plasma membrane NAPDH oxidases (Daudi et al., 2012). Clade I TGAs have been shown to contribute to this ROS burst upon pathogen perception in Arabidopsis (Boller & Felix, 2009; Wang & Fobert, 2013). To test if BOP1 and BOP2 also contribute to this burst, hydrogen peroxide production was measured in wild-type, *BOP1-oe, bop1 bop2, tga1 tga4, bop1 bop2 tga1 tga4* leaf disks exposed to flg22 peptide,

a 22-amino-acid fragment of bacterial flagellin that elicits PTI. Leaf disks exposed to water were used as a negative control.

An ROS burst was swiftly induced in wild-type plants, peaking at about 6 minutes. Compared to the wild type, *bop1 bop2* mutant plants showed no reduction in ROS production, but ROS production was both diminished and delayed in *tga1 tga4* double and *bop1 bop2 tga1 tga4* quadruple mutants. *BOP1-oe* leaves generated a significantly higher amount of ROS compared to wild type (Figure 2-5). These data indicate that BOP1/2 are not crucial for ROS production but BOP1 can stimulate this response when overexpressed.

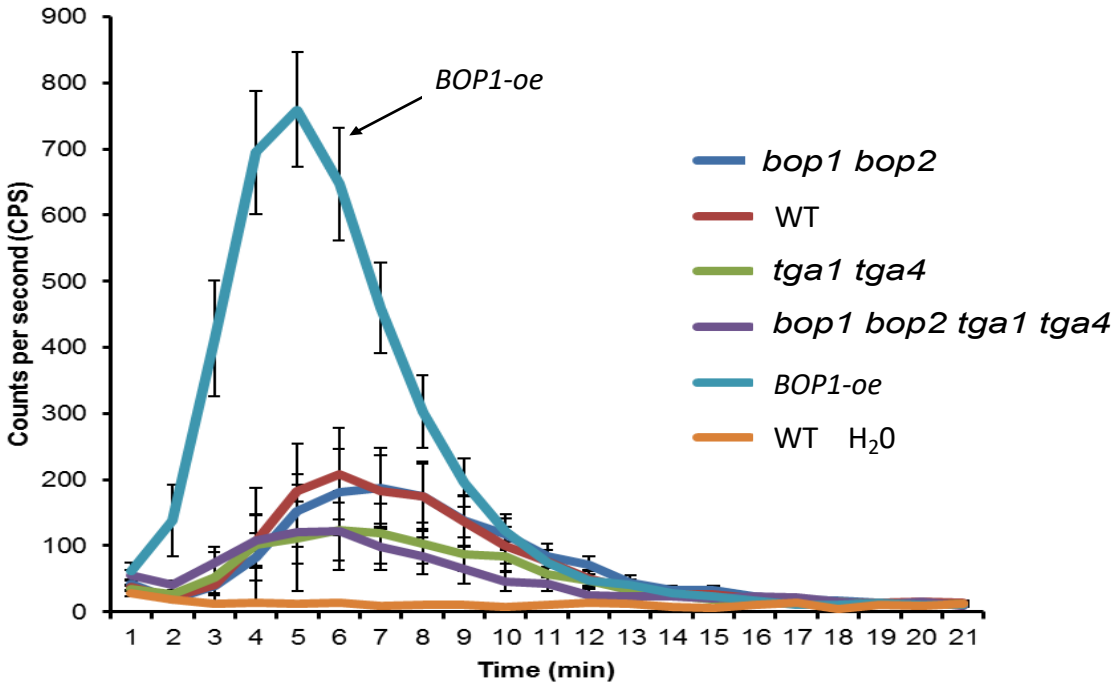


Figure 2-4. PAMP-induced oxidative burst in wild type, *BOP1-oe, bop1 bop2, tga1 tga4* and *bop1 bop2 tga1 tga4* plants.

Four-week-old leaf discs (2 per each sample) were treated with or without 100 nM flg22 in the presence of luminol, and the H_2O_2 generated was measured. The error bars represent SE of 8 replicates. Representative of three independent trials.

2.4.2.3 Callose deposition assay

Another component of PTI is the accumulation of polymers that strengthen the cell wall in areas of pathogen attack. Notable is the deposition of papillae containing the β 1,3 glucan callose (Gómez-Gómez & Boller, 2000; Hématy et al., 2009). Callose deposition was induced by infiltrating wild-type, *BOP1-oe*, *bop1 bop2, tga1 tga4*, and *bop1 bop2 tga1 tga4* leaves with *Pst hrcC*$^{-}$ or flg22 peptide to activate a defense response. Leaves infiltrated with a mock solution were used a negative control. Callose deposits were monitored after 24 hours by staining with aniline blue.

A large number of callose deposits were observed in wild-type leaves treated with *Pst hrcC*$^{-}$ or flg22. The number of depositions was significantly increased in *BOP1-oe* leaves by ~1.8-fold compared to wild-type (Figure 2-6). In contrast, the leaves of *bop1 bop2, tga1 tga4,* and *bop1 bop2 tga1 tga4* mutants showed significantly fewer callose deposits (3 to 4-fold lower than wild-type) following treatments with *Pst hrcC*$^{-}$ or flg22. Little or no callose was deposited in leaves treated with water as a negative control. These data indicate that *BOP* genes and clade I TGA factors are required for *Pst hrcC*$^{-}$ and flg22 induced callose deposition.

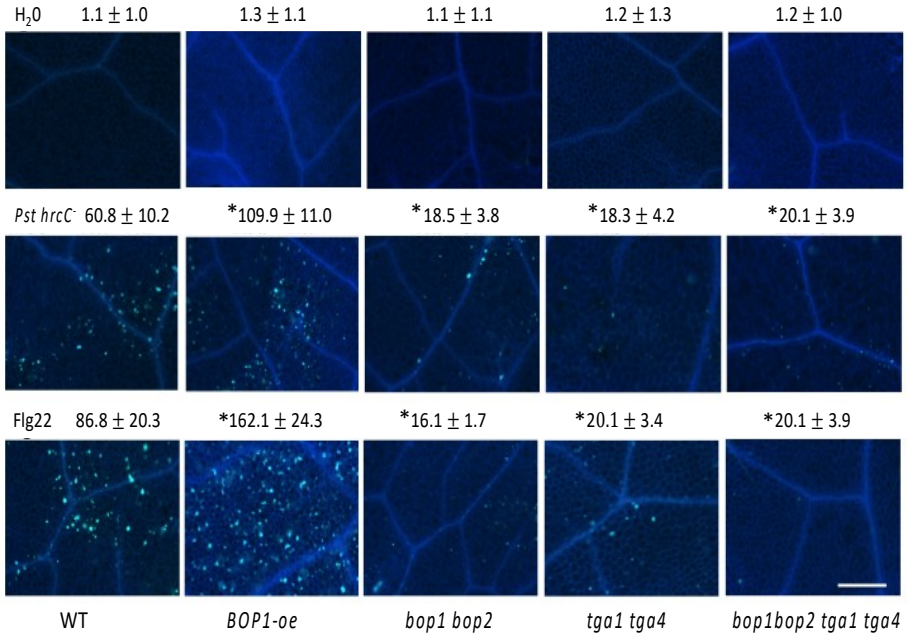


Figure 2-5. MAMP and pathogen induced callose deposition in wild type, *BOP1-oe, bop1 bop2, tga1 tga4* and *bop1 bop2 tga1 tga4* plants.

Values above each picture represent the mean ± SD of the number of callose depositions, based on three plants per mutant, three leaves per plant, and three sectors per leaf = 27 sectors per genotype and treatment. Callose deposits were numerous in flg22 and *Pst hrcC*$^-$ treated leaves of *BOP1-oe* and Col-0 plants. In leaves of *bop1 bop2, tga1 tga4 and bop1 bop2 tga1 tga4*, relatively few callose deposits were observed following flg22 treatment and *Pst hrcC*$^-$ challenge. Asterisks indicate significant difference compared to WT control ($p \leq 0.001$, Student's t-test). Scale bar = 0.1 mm (all photos are at the same magnification). Images are representative of three independent trials.

2.4.2.4 Investigating defense related production of proteins in *BOP1-oe, bop1 bop2* and wild type leaves.

A third hallmark of PTI is the production of extracellular proteins that strengthen the cell wall or have antimicrobial activities (Kwon et al., 2008). To examine this response, I monitored the apoplastic accumulation of PR1 protein via immunoblotting of intercellular washing fluids (IWFs) following infection with *Pst hrcC*$^-$ (Wang & Fobert, 2013). Leaves of four-week-old wild-type, *BOP1-oe* and *bop1 bop2* plants grown under short days were

syringe-infiltrated with 10^8 cfu ml^{-1} of *Pst hrcC*$^-$. An *npr1* mutant was used as a negative control. Leaves from three plants per genotype were collected and pooled as one sample for intercellular washing fluids (IWFs) protein extraction. Samples were collected at 0, 1 and 2 days post inoculation (dpi) and analyzed by Western blotting with a PR1 antibody.

My results showed that in response to *Pst hrcC*$^-$ challenge, PR1 protein was detectable in IWFs from *BOP1-oe* leaves at one hour after pathogen inoculation and measurable 2 dpi. Compared to the wild type, no significant deviation was noticed in *bop1 bop2* mutant leaves as both showed very low levels of protein accumulation at 2 dpi compared to *BOP1-oe* (Figure 2-7). These data indicate that BOP1/2 are not crucial for PR1 protein expression but BOP1 can stimulate this response when overexpressed.

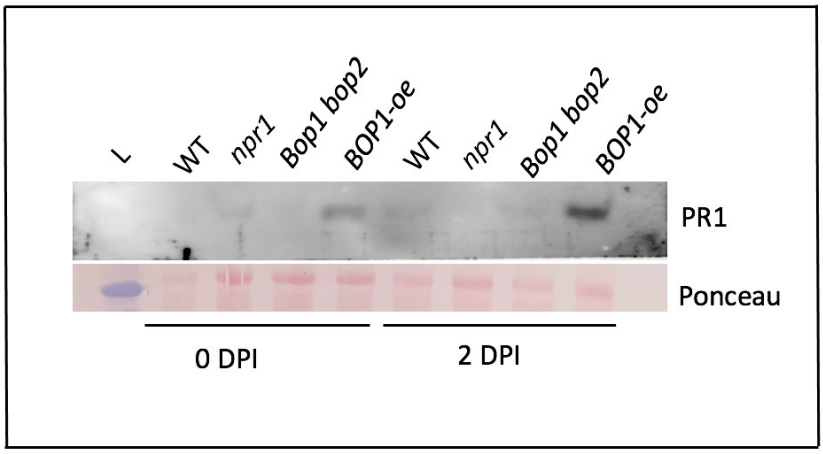


Figure 2-6. Upper panel, PR1 protein accumulation in Col-0 WT, *npr1, bop1 bop2,* and *BOP1-oe* following *Pst hrcC*$^-$ inoculation.

Samples were obtained 1 hour after pathogen inoculation (0 DPI), Day 2 samples were collected at 48 hours post inoculation (2 DPI). PR1 protein, ~17kDa. Lower panel, same gel stained with Ponceau stain as a loading control. Purple band, L (50 KDa). Experiment was done once.

2.5 Discussion

Plants use systems of innate and induced immunity to detect and repel invading pathogens (Jones & Dangl, 2006). The activation of these defenses is tightly linked to growth repression as a way of balancing the use of limited resources (Huot et al., 2014). BOP1 and BOP2 belong to a family of co-transcription factors with roles in growth and defense. While NPR1-like members are essential for systemic acquired resistance, BOP1-like members regulate plant morphology (Backer et al., 2019; Khan et al., 2014). Despite these differences, BOP1/2 and NPR1 share homologous BTB/POZ and ankryin functional domains and both types of proteins interact with defense-related TGA bZIP transcription factors to exert their function (Chen et al., 2019; Després et al., 2003; Hepworth et al., 2005; Saleh et al., 2015; Wang et al., 2019; Zhang et al., 2003; Zhang et al., 2019). An initial characterization of the *bop1 bop2* mutant found no obvious change in resistance to *Pseudomonas syringae maculicola* ES4326 (Hepworth et al., 2005). Later studies found that *bop1 bop2* mutants lack a priming response to MeJA and alter the responsiveness of *npr1 npr2* and *nrp3 npr4* mutants to SA treatments, suggesting an involvement in JA or SA defense responses (Canet et al., 2012; Canet et al., 2010; Castelló et al., 2018). A recent study on cotton plant, *Gossypium hirsutum,* found that GhBOP1 was transiently activated outside of organ boundaries in the stem to control lignification as a defense response to *Verticillium dahliae* (Zhang et al., 2019). Consequently, the function of BOP1/2 in resistance to pathogens needs further evaluation.

Here, fungal and bacterial pathogens were used to examine the involvement of BOP1/2 in plant defense. I found that plants with overexpression of BOP1 were more resistant to both types of pathogen whereas *bop1 bop2* mutants were more susceptible.

Further analysis identified a specific role for BOP1/2 in pattern-triggered immunity. These data expand our knowledge of BTB-ankryin proteins and their many roles in plant defense.

2.5.1 Plants overexpressing BOP1 prioritize defense over growth

BOP1 and BOP2 in the NPR1 family regulate development at organ boundaries where growth is restricted (Khan et al., 2014). An obvious consequence of overexpression of these genes is a dwarf phenotype. Here, *BOP1*-overexpressing (*BOP1-oe*) plants were characterized to show broad-spectrum immunity to pathogens. The infiltrated leaves of *BOP1-oe* plants showed greater resistance to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, a biotrophic bacterium that elicits a strongly SA-dependent defense response (Kim et al., 2008) as well as *Botrytis cinerea*, a fungal necrotroph that elicits a strong JA/ET-dependent defense response (Windram et al., 2012). Autoimmunity is a condition that leads to enhanced disease resistance through the constitutive expression of defense genes. Other typical phenotypes include dwarfism, high resting levels of salicylic acid, and in some cases, high resting levels of ROS that lead to spontaneous cell death lesions (van Wersch et al., 2016). Autoimmune mutants often contains lesions that increase innate immunity, which is effective against diverse pathogens (van Wersch et al., 2016). Indeed, microarray analysis of *BOP1-oe* plants found that a majority of upregulated genes classified as "biotic stress-related" were associated with PTI (55.7%) ahead of JA/ET (49.8%) and SA (33.7%) defense-related pathways (Wang et al., 2020). Investigation of PTI-related genes by RT-qPCR found that transcripts for the flagellin receptor gene *FLS2* (Zipfel et al., 2004) and co-receptor gene *BIK1* (Lu et al., 2010) were upregulated 3.0- and 4.5-fold respectively. The respiratory burst oxidase gene *RBOHD* that contributes to ROS

production (Morales et al., 2016) and callose synthase gene *GLS5* (Jacobs et al., 2003) were also significantly up-regulated as were clade I TGA regulated genes *SARD1* (up-regulated 3.9-fold) and *CPB60g* (up-regulated 2.5-fold) which promote SA biosynthesis (Sun et al., 2015; Sun et al.,2 018). In agreement, *BOP1-oe* leaves infected with *Pst hrcC*$^{-}$ to assay PTI showed significantly lower bacterial titres compared to the wild-type (this thesis). This resistance was associated with a stronger oxidative burst, increased callose deposition, and high resting levels of PR1 protein secretion (this thesis). Despite this increase, resting levels of ROS, callose, and SA accumulation appeared to be normal (Wang et al., 2020).

2.5.2 Loss of function *bop1 bop2* plants have impaired disease resistance

A double mutation of *BOP1* and *BOP2* dramatically impairs the patterning of organ boundaries where these genes are highly expressed. Lower abundance transcripts are found in leaves, vascular tissue, and roots as evidence of a broader role (Hepworth et al., 2005; Woerlen et al., 2017; http://bar.utoronto.ca/). It was earlier reported that *bop1 bop2* leaves infiltrated with *Pseudomonas syringae maculicola* ES4326 showed no obvious change in resistance compared to the wild type (Hepworth et al., 2005), nor was a significant difference in pathogen growth observed in spray treatment of *bop1 bop2* seedlings with *Pseudomonas syringae* DC3000 (Canet et al., 2012). In this study, mature *bop1 bop2* leaves infiltrated with *Pseudomonas syringae* DC3000 showed significantly higher titres of bacteria compared to the wild type in three independent trials. Differences in growth conditions, the pathogen strain, or how/when disease symptoms were monitored might account for these contrasting results. Leaves of the *bop1 bop2* mutant treated with fungal spores of the necrotroph *B. cinerea* also showed greater disease progression than the wild

type, showing that sensitivity of the *bop1 bop2* mutant is not limited to pathogens of one particular lifestyle. Mutation of the PAMP receptor protein CERK1 also impairs disease resistance to *Pseudomonas syringae* and fungal pathogens (Zhang & Zhou, 2010).

2.5.3 BOP1/2 and clade I TGAs have partially overlapping effect on PTI

BOP1 and BOP2 carry out developmental functions with TGA transcription factors (Hepworth et al., 2005; Wang et al., 2019). Among these partners, clade I TGA1 and TGA4 also function in PTI (Wang & Fobert, 2013). The *tga1 tga4* mutant is compromised in defense against *Pst hrcC*$^{-}$ and oxidative burst, callose deposition and PR1 protein secretion (Wang & Fobert, 2013). My data provide evidence that BOP1/2 and clade I TGAs have overlapping functions in PTI. *Pst hrcC*$^{-}$ grew to similar titres in *bop1 bop2* and *tga1 tga4* leaves and was not further increased in the *bop1 bop2 tga1 tga4* quadruple mutant, showing that combining *bop1 bop2* and *tga1 tga4* mutations did not worsen disease symptoms. Callose depositions in the infected leaves of *bop1 bop2*, *tga1 tga4* and the quadruple mutant were also comparable, showing an ~70% reduction compared to the wild type. No significant change in the oxidative burst or PR1 secretion was observed in *bop1 bop2* mutants, suggesting that BOP1/2 are not crucial for these responses.

We consider it likely that BOP1/2 and clade I TGAs co-regulate a subset of genes involved in the PTI response. Good candidates for this co-regulation are the transcription factors genes *SARD1* and *CBP60g*, which are involved in the production of salicylic acid (Sun et al., 2015; Sun et al.,2 018). The expression of these genes is reduced in pathogen-treated *tga1 tga4* plants (Sun et al., 2018) and in flg22-treated *bop1 bop2* and *tga1 tga4* seedlings compared to wild-type seedlings (Wang et al., 2020). The upstream non-coding

regions of these genes contain numerous motifs that resemble TGA binding elements. TGA1 was shown by chromatin immunoprecipitation to bind directly to a TGACG-containing region in the *SARD1* promoter whereas the regulation of *CBP60g* might be indirect (Sun et al., 2018). Chromatin-immunoprecipitation experiments using an epitope-tagged BOP1 protein showed binding to TGACG-containing regions of both promoters including the same region that showed TGA1 binding. Interestingly, this binding was only observed in flg22-treated seedlings, suggesting that BOP1 does not bind constitutively to these promoters (Wang et al., 2020). RNA sequencing was used to examine the transcriptomes of flg22-treated wild-type, *bop1 bop2,* and *tga1 tga4* seedlings. This experiment identified a larger pool of 378 genes that were significantly induced in wild-type seedlings but showed little or no change in *bop1 bop2* and *tga1 tga4* seedlings. Among these co-regulated genes were seven WRKY defense-associated transcription factor genes (Eulgem, 2005), *PAD4* required for the production of phytoalexin defense compounds (Glazebrook et al., 1997), two CC-type glutaredoxin genes involved in the redox-modification of TGA transcription factors (Murmu et al., 2010; Zander et al., 2012) and ten oxidase, laccase, and peroxidase genes presumably involved in ROS production and/or cell wall thickening (Lee et al., 2019; O'Brien et al., 2012). Further analysis of these genes can be used to clarify the joint role of BOP1/2 and clade I TGA factors in plant immunity.

CHAPTER 3: Investigating the role of hydrophobic cell-wall polymer suberin in *Arabidopsis thaliana* defense against bacterial and fungal pathogens

3.1 Abstract

In this chapter, I used Arabidopsis to examine the role of the hydrophobic cell-wall polymer, suberin, in protecting the plant against pathogenic bacterium *Pseudomonas syringae hrcC*$^{-}$ and the fungi *Fusarium avenaceum* and *Botrytis cinerea*. Suberin is deposited at the cell wall as a protective barrier against abiotic and biotic environmental stresses in various tissues of terrestrial plants. It can occur as a preformed or induced complex of extracellular, hydrophobic, lipid-based barriers and it plays important roles in controlling water and ion movement. For my experiments, mutants of *A. thaliana*, defective in root suberin were used in pathogen test procedures. The suberin mutants used were defective in suberin monomer biosynthesis (*cyp86a1-1 cyp86b1-1* and *far1-2 far4-1 far5-1*), or suberin transport (*abcg2-1 abcg6-1 abcg20-1*) or regulation (*myb92-1 myb93-1*). As well, mutants were used that ectopically express suberin in the leaves and tested for resistance or susceptibility to the pathogens. My findings indicate that suberin deposition in Arabidopsis helps prevent water and fluid loss from the root. Strikingly, I found that the presence of suberin in the leaves of Arabidopsis promotes water loss in the aerial surface of the plant. I found preliminary evidence of increased susceptibility of suberin mutants using the pathogen assays. The interpretation of these susceptibility tests is complicated, however, by other physiological defects of suberin mutants, in particular, water balance perturbations associate with suberin mutants.

3.2 Introduction

3.2.1 Suberin polymer and defense against plant pathogens

Many tissues of higher plants accumulate suberin, including roots, seed coats, tree bark, the periderm of mature roots and tubers, and endoderm of young roots (Beisson et al., 2012; Franke et al., 2012; Pollard et al., 2008). Suberin is a hydrophobic acylglycerol polymer deposited on the inner surface of the cell wall of certain types of cells, where it is thought to have important functions in plant water relations and in protecting plants from abiotic stresses such as drought, high salinity and wounding (Philippe et al., 2020).

There have been many speculations that suberin acts as a barrier to restrict pathogen invasion in plants, and suberin is also considered to be a wound-induced anti-microbial component in defense responses against pathogens (Vishwanath et al., 2013). Lulai & Corsini (1998) were the first to show that suberization of wounded potato tubers offered protection against infection by bacterial strains of *Erwinia carotovora* and the fungus *Fusarium sambucinum*. It was shown that development of resistance to bacterial and fungal penetration was related to differential deposition of the two major suberin domains, polyphenolic and polyaliphatic, during wound healing. Bernards & Lewis (1992) proposed that the polyphenolic domain determines the function of suberin as a barrier to pathogen attack. Thomas et al. (2007) established that preformed suberin is present in both the endodermis and epidermis of soybean root and showed a positive correlation between soybean root suberin content and partial resistance to root rot caused by *Phytophthora sojae*. However, contrary to Bernards & Lewis (1992), the strongest relationship between the amount of suberin in the roots of soybean and partial resistance to *P. sojae* was observed with the aliphatic component of the polymer.

Indeed, manipulating suberin deposition may have applications in designing pathogen resistance in plants (Churchward et al., 2018; Ranathunge et al., 2008), and may lead to design of new antimicrobials. In the later possibility, a recent publication by Correia et al. (2020) isolated suberin from cork via mild cleavage of acylglycerol esters catalyzed by cholinium hexanoate. This method left the glycerol bonds that safeguard the polymeric nature of suberin intact, or only partially cleaved. The purified suberin has promising attributes since it is non-toxic to animals (Coquet et al., 2005) and displays inhibitory activity against the human pathogenic bacteria, *Staphylococcus aureus* and *Escherichia coli.*

However, studies on root suberin as a barrier to restrict pathogen invasions by direct pathogen tests are rare. Therefore, the precise roles of suberin and associated waxes in defending against fungal and bacterial pathogens are surprisingly poorly documented. It is crucial to research and document these potential protective functions of suberin, as a better understanding of these could pave the way for new strategies that employ suberin for the development of more pathogen resistant crop plants. Ultimately, this would help improve the performance of crop plants and increase global food security. This chapter explores the roles of suberin in protecting Arabidopsis against invasion by fungal and bacterial pathogens, and against uncontrolled water loss from the roots and leaves to the environment.

3.2.2 Mutations affecting suberin deposition

Mutations in the genes encoding enzymes involved in the biosynthesis of suberin lead to reduced amounts of suberin or altered composition of suberin. Arabidopsis plants

with suberin mutations have various phenotypes such as increased seed permeability, increased root system hydraulic conductivity, and seed germination sensitivity to abscisic acid (Franke et al., 2012). The Rowland lab (Carleton University) characterized biosynthesis of suberin-associated primary fatty alcohols and have developed a series of single, double and triple fatty acyl reductase *(far)* mutants affected in suberin-associated fatty alcohols and suberin-associated root waxes (Domergue et al., 2010; Vishwanath et al., 2013). Fatty alcohols of chain lengths C18, C20 and C22 are components of Arabidopsis root suberin (Domergue et al., 2010; Vishwanath et al., 2013), and the enzymes fatty acyl reductases (FARs) catalyze the reduction of fatty acyl-CoAs to primary fatty alcohols through an unreleased fatty aldehyde intermediate (Rowland & Domergue 2012). There is an eight-member gene family of FARs in Arabidopsis. I tested *Fusarium avenaceum* infections in the far triple mutants (*far1-2 far4-1 far5-1*), which showed ~70% reduction in total fatty alcohol load in root and seed coat suberin (Vishwanath et al., 2013).

Cytochrome P450 CYP77A, CYP86A, and CYP86B family members are involved in fatty acid oxygenation. P450 fatty acid ω-hydroxylase CYP86A1 (At5g58860) is vital in the biosynthesis of aliphatic root suberin in Arabidopsis (Höfer et al., 2008). *cyp86a1/horst* knock out mutants, which have about 60% less total aliphatic root suberin and deformed lamellae structure, are primarily affected in C16 and C18 hydroxyacids and α, ω-dicarboxylic acids, compared to wild type plants, and were found to have a reduced apoplastic barrier to the radial flow of water and NaCl in the root (Ranathunge & Schreiber, 2011). *cyp86b1*/*ralph* mutations result in reductions of C22- and C24-hydroxyacids and α, ω-dicarboxylic acids in root and seed coat aliphatic polyesters (Compagnon et al., 2009).

The double knockout mutant, *cyp86a1-1 cyp86b1-1* (also known as *ralph-1 horst-1*), was tested for resistance or susceptibility to the fungal pathogen, *Fusarium avenaceum*.

ATP-binding-cassette (ABC) transporters of the G-subfamily located in the plasma membrane are believed to be involved in suberin monomer transport across the plasma membrane to the suberin assembly site (Vishwanath et al., 2015). It was also shown that ABCG1 is necessary for the formation of suberin in potato tuber periderm (Landgraf et al., 2014). In Arabidopsis, the *abcg2-1 abcg6-1 abcg20-1* triple mutant was shown to have alterations in the suberin lamellae structure, composition and properties of root and seed coat suberin, and the root system of the mutant plants were more permeable to water and salt (Yadav et al., 2014). I used this triple mutant in my plant pathology assays.

MYB-type transcription factors contain conserved MYB DNA-binding domains and, in Arabidopsis, are believed to be involved in the regulation of the specific developmental and stress-induced gene expression patterns of the suberin biosynthetic genes (Vishwanath et al., 2015). According to Dubos et al. (2010), Arabidopsis has over 100 genes encoding MYB transcription factors that may regulate many plant-specific processes such as biosynthesis of anthocyanin and secondary cell walls. The Rowland lab explored the involvement of MYB in suberin biosynthesis using Arabidopsis and *Nicotiana benthaminana*. It was discovered that in both species, under abiotic stress conditions, AtMYB41 (At4g28110) is a transcriptional factor that is able to activate important steps in the biosynthesis of aliphatic suberin and exportation, assembly and deposition of cell wall associated suberin-like lamellae (Kosma et al., 2014). *35S* promoter-driven overexpression of *AtMYB41* (*AtMYB41 OE-9*) leads to increases in abundance of suberin biosynthetic gene transcripts, which causes accumulation of more suberin-type than cutin-type aliphatic

monomers in plant leaves (Cominelli et al., 2008; Kosma et al., 2014). The Rowland lab generated additional *AtMYB41 OE*- overexpression lines that at four weeks of age accumulate aliphatic suberin content in the leaves that are one to three times that of their cutin monomer content. For example, *AtMYB41 OE-63* accumulates the highest aliphatic suberin, almost three times the cutin monomer content, *AtMYB41 OE-7* and *AtMYB41 OE-10* accumulates about two times, while in the leaves of *AtMYB41 OE-11* and *AtMYB41 OE-12,* there were no suberin accumulated (negative controls). The amount of total cutin monomers were similar across all the generated lines with the exception of one line, *AtMYB41 OE-63*, which had slightly less total cutin than the wild type.

It was also discovered that AtMYB92 and AtMYB93 have overlapping gene expression patterns in root endodermis, and *myb92 myb93* T-DNA knockout lines showed significantly decreased suberin deposition in total root suberin, and major monomer contents of about 40% compared to wild type at four weeks (Rowland lab-unpublished data). To test suberin as a structural barrier against fungal pathogen in the root of Arabidopsis, the double mutant was used. *AtMYB41 OE* lines generated by the Rowland lab (*AtMYB41 OE-7*, *AtMYB41 OE-10, AtMYB41 OE-11*, *AtMYB41 OE-12 and AtMYB41 OE-63*) are also used in this study to see whether or not ectopically expressed suberin serves as a barrier to invasion by leaf pathogens.

3.2.3 Rationale behind suberin studies

The availability of mutants of various genes of Arabidopsis that each have unique alterations in root suberin abundance and composition represents an opportunity to study the possible roles of suberin in protecting plants against pathogen attack. There have been

no experiments reported previously that test how *A. thaliana* suberin mutants respond to pathogenic microorganisms. Therefore, in this study, I focused on investigating Arabidopsis Columbia (Col-0) wild type and suberin mutants with altered amounts of suberin for disease susceptibility and/or resistance to fungal and bacterial pathogens.

- Plant host systems – *A. thaliana* Col-0 wild type, suberin mutants that are altered in suberin characteristics.
- Pathogen systems –Plant root fungal pathogen *Fusarium avenaceum*, leaf fungal pathogen *Botrytis cinerea* and bacterial pathogen, *Pseudomonas syringae pathovar* tomato.

Projects:

- Investigating the roles of Arabidopsis root suberization in protection against a plant root fungal pathogen.
- Investigating the roles of ectopic suberin expression in Arabidopsis leaves in defense against bacterial and fungal pathogens.

Hypotheses:

1. Cell wall suberization acts as barrier to the invasion of plant root fungal pathogens Prediction: mutant plants deficient in root suberin deposition will have increased susceptibility to the pathogens, compared to the wild type.
2. Cell wall suberization in root endoderm and or periderm acts as barrier to the loss of water via the roots of *A. thaliana.* Prediction: mutant plants deficient in root suberin deposition will have increased water loss via the roots in comparison to the wild type.
3. Ectopic suberin expression in Arabidopsis leaves acts as barrier to the invasion of

leaf bacterial and fungal pathogens. Prediction: mutant plants ectopically expressing suberin will have increased resistance to leaf bacterial and fungal pathogens, compared to the wild type.

4. Ectopic suberin expression in Arabidopsis leaves acts as barrier to loss of water through the leaf surfaces. Prediction: mutant plants ectopically expressing suberin will have reduced water loss through the surface, compared to the wild type.

Research objectives and methodology

I investigated the resistance or susceptibility of suberin mutants to a root pathogenic fungus, as well as leaf fungal and bacterial pathogens. I also examined and compared water loss through the roots of Arabidopsis mutants and wild type, as well as leaves of plants with ectopically expressed suberin. This entails culturing selected pathogens, development of suitable pathogen assays by infecting plants with fungal pathogen strains, and monitoring disease symptoms in Arabidopsis wild type and suberin mutants. I evaluated disease severity based on plant morphology, size, growth restriction, and chlorosis in comparison to control plants.

3.3 Materials and method

3.3.1 Plant material and growth conditions

Arabidopsis seeds of the Columbia-0 (Col-0) ecotype was used as wild type. Arabidopsis suberin mutants were graciously provided by Rowland lab, including double mutant *myb92-1 myb93-1* (Murmu et al., unpublished), double mutant *cyp86a1-1 cyp86b1-1* (also known as *ralph-1 horst-1*) (Compagnon et al., 2009), triple mutant *abcg2-1 abcg6-1 abcg20-1* (Yadav et al., 2014), triple mutant *far1-2 far4-1 far5-1* (Domergue et al., 2010; Vishwanath et al., 2013; Yadav et al., 2014), and *AtMYB41 OE-7*, *AtMYB41 OE-10, AtMYB41 OE-11*, *AtMYB41 OE-12* and *AtMYB41 OE-63* (Cominelli et al., 2008; Kosma et al., 2014).

Seeds were rinsed in 100% ethanol and then transferred to a freshly made solution of 5% sodium hypochlorite and 0.5% (w/v) sodium dodecyl sulphate (SDS) for one minute of incubation. Seeds were then rinsed four times in sterile distilled water and sown on agar plates containing minimal media (Haughn & Somerville, 1986) for plant propagation, on 1% Murashige and Skoog (MS) medium for experiments involving seedlings, or directly in trays of steam-sterilized soil (ProMix BX, Premier Horticulture, Riviere-du-loup, QC) supplemented with a 1 g L^{-1} solution of 20-20-20 plant fertilizer (Plant-Prod Inc., Brampton, ON) for other experimental procedures. Plated seeds were wrapped in foil and incubated at 4ºC in the dark for 2-3 days to break dormancy, while seeds on steam-sterilized soil were covered with black trays and kept in the cold room for 2-3 days to break dormancy. Following cold treatments, both plates and trays for experiments were moved to the growth chamber under 16 hours light for 9-10 days for seed germination. Seeds on plates to be used for propagation were moved to growth chambers under continuous light (~115 µmol m^{-2} sec^{-1}) at 21ºC. After germination, seedlings for propagation were transplanted from

plates to trays of steam-sterilized soil (ProMix BX, Premier Horticulture, Riviere-du-loup, QC) supplemented with a 1 g L^{-1} solution of 20-20-20 plant fertilizer (Plant-Prod Inc., Brampton, ON), and grown to maturity in growth chambers under continuous light (~115 µmol m^{-2} sec^{-1}) at 21ºC. Seedlings for experiments were grown until four weeks old in growth chambers under long days (16 h light/8 h dark) at 21-22ºC (~115 µmol m^{-2} sec^{-1}).

3.3.2 Pathogen cultivation and maintenance

The *hrcC*$^{-}$ mutant (*Pst hrcC*$^{-}$) of *Pseudomonas syringae Pst* DC3000 (*Pst hrcC*$^{-}$*)* was kindly provided by Gopal Subramaniam at Agriculture and Agri-Food Canada, and maintained as described by Ritter & Dangl (1996). *Pseudomonas syringae* was grown, inoculated and assayed for growth on the plant as described by Tornero & Dangl (2001). *Botrytis cinerea* strain RW1A6P and *Fusarium avenaceum* (EM RW1A5P) were both isolated from rotting wood in Ottawa, Ontario and identified to species by ITS sequence and morphological characteristics (Micalizzi et al., 2017). Both strains were maintained on potato dextrose agar (PDA).

3.3.3 Pathogen assays

3.3.3.1 *Fusarium avenaceum* assay

Plants were assayed for resistance to *Fusarium avenaceum* infection according to Edgar et al. (2006) and Chen et al. (2014). In brief, spores of *F. avenaceum* strain RW1A6P were harvested from potato dextrose agar (PDA) plates into sterile water. The spore suspension was filtered through four layers of sterile cheesecloth and washed twice in sterile water. The spores were resuspended into sterile water and diluted to a concentration

of 10^8 spores ml^{-1} for pathogenicity tests. Four-week-old plants were gently uprooted and dipped for 15 seconds in fungal spore suspension and then replanted into a new pot. Mock treatments involved dipping plants in water without spores. Indications of plant disease were monitored daily for two weeks, and photographs were taken at indicated time intervals. Also, four-week-old plants (12 plants per mutant plus of the wild type) were gently uprooted and replanted, then challenged with four 6 mm diameter plugs from the colony margin of 6-8-day old cultures of *F. avenaceum* at the root area. Indications of plant disease were monitored daily for two weeks, and photographs were taken at indicated time intervals. Alternately, seed plants were grown directly on 1% Murashige and Skoog (MS) medium and challenged with 6 mm diameter plugs from the colony margin of 6-8-day old cultures of *F. avenaceum*.

3.3.3.2 *Botrytis cinerea* assay

Plants were assayed for resistance or susceptibility to *Botrytis cinerea* infection as described by (Murmu et al., 2014). Spores of *B. cinerea* strain RW1A6P were harvested from potato dextrose agar (PDA) plates into sterile water. The spore suspension was filtered through four layers of sterile cheesecloth and washed twice in sterile water. The spores were resuspended in potato dextrose broth (PDB) and diluted to a concentration of 5×10^5 ml^{-1} for pathogenicity tests. Detached leaves from four-week-old plants grown under long days (16 h light/8 h dark) at 22ºC were placed separately on three layers of moist sterile Whatman filter paper (Fisher Scientific, ON, Canada) inside plastic Petri dish. The center of each leaf was inoculated with 6 µL of the spore suspension. The plates were sealed with parafilm to maintain humidity and incubated in the dark for the first 12 hours and then

transferred to a growth chamber set at 22°C long days (16 h light/8 h dark) and monitored for ten days. The diseased leaves were photographed at various times to record lesion sizes. Lesion sizes were measured from the photographs using ImageJ software (http://www.nih.gov).

3.3.3.3 *Pseudomonas syringae* assay

Plants of *AtMYB41 OE* that express suberin ectopically were assayed for resistance against the *hrcC*$^{-}$ mutant of *Pst* DC3000 (*Pst hrcC*$^{-}$) as described (Canet et al., 2012; Shearer et al., 2012; Wang & Fobert, 2013). In brief, bacteria were grown at 28°C on King's B agar medium containing Rifampicin (100 mg l^{-1}). Overnight cultures were resuspended in infiltration medium (10 mM $MgCl_2$). Leaves of four-week-old plants grown under long days (16 h light/8 h dark) were sprayed with bacteria at 10^8 colony-forming units (cfu) ml^{-1} in 0.02% Silwet L-77 (CromptonEurope Ltd, Evesham, UK). Plants were monitored for three days in the growth chamber. To determine the bacterial growth in Arabidopsis leaves, bacterial numbers were measured at several time points (0, 1, 2, 3- and 4-days post inoculation (dpi)). The bacterial numbers were evaluated from three replicates per genotype, and each replicate consisted of 4 leaf discs (1 cm^2) from the infected leaves of four different plants. To enumerate, leaf samples were homogenized in sterile water, and bacterial titers were determined by plating serial dilutions of the homogenized samples on King's *B agar* medium containing rifampicin (100 mg l^{-1}) agar. Two days after plating of diluted samples, the average number of the bacterial colonies were determined to obtain colony forming units (CFU) per cm^2 of leaf area. Averaged CFU values were log-transformed and statistically analyzed.

3.3.4 Measurement of root periderm segments for water loss

The method described in Bu et al. (2014) was used to measure water loss from mature root periderm segments of suberin mutants and with wild type at 15 minutes intervals for about 90 minutes. A ~1 cm segment was cut from the tap root at the base of the root system of four-week-old plants. To prevent rapid water loss, a thin layer of Vaseline was applied to the cut surfaces and samples were weighed immediately. Subsequent weights were taken at 15 minutes intervals for 75 minutes. The loss of mass due to water loss was calculated as a percentage of the initial mass.

3.3.5 Toluidine Blue O Staining

The permeability of leaves of *AtMYB41 OE* mutant transgenic lines and wild type for Toluidine Blue O (TBO) was assayed according to Tanaka et al. (2004) and Mahmood et al. (2019). Briefly, leaves with cuticle that is not intact should display intense blue staining with TBO because of fast infiltration of the stain into the leaf, whereas leaves with intact cuticle will not be stained because the cuticle should resist the dye movement into the leaf. Leaves from three to four-week-old plants were detached from wild type and *AtMYB41 OE* mutant transgenic lines and submerged in an aqueous solution of 0.05% (w/v) TBO for two to five minutes. The TBO solution was then removed from the leaf samples and leaves were washed gently with distilled water to remove excess TBO. Leaves of three to four whole rosettes were immersed in the staining solution representing each genotype.

3.3.6 Chlorophyll Leaching Assay

Chlorophyll leaching assay of rosette leaves was performed as described by Xia et al. (2010). Briefly, leaves of *AtMYB41 OE* mutant transgenic lines and wild type were obtained from four-week-old plants grown in soil and weighed. Initially, all genotypes had the same green colour to the leaves. 100 mg of leaves were incubated in tubes containing 80% ethanol in the dark at room temperature with gentle shaking. Samples of the ethanol solution were taken at several time points for each genotype and absorbance of each sample was measured at both 664 and 647 nm. Micromolar concentrations of total chlorophyll per gram fresh weight in each of the samples were calculate by using the equation:

Total chlorophyll (μmoles/mg fresh weight of sample) = 7.93 (A664) + 19.3 (A647).

3.3.7 Transpirational water loss

For measurement of water loss via transpiration, the method described by Cominelli et al. (2008) was employed. In brief, detached rosette leaves of 3-week-old plants grown on soil were placed on 3 MM filter papers (Whatmann) within 9 cm Petri dishes at 22°C for the indicated time periods. The degree of dehydration was measured by comparing the fresh weight (FW) of the leaves before and after the dehydration treatment. The assay was performed in triplicate. Ten plants were used for each time point in each assay.

3.3.8 Relative Water Content (RWC) determination

At 4 weeks of age, five 6 mm discs were collected from fully developed leaves (one disc per leaf) of pathogen infected plants and control to determine the leaf relative water

content as previously described (Pieczynski et al., 2013). The leaf discs were weighed immediately after removing from the plant to determine the fresh mass (FM). These leaf discs were then hydrated by floating in water for 4 hours at the room temperature, after which the 'saturated mass' of the leaf discs (SM) were weighed after surface drying with paper towel. Leaf discs were then dried for 36 h at 60°C and reweighed to determine the dry mass (DM). The relative water content (RWC) was calculated from leaves from five individual plants per genotype as:

$$RWC = (FM\text{-}DM)/(SM–DM) \times 100$$

3.4 Results

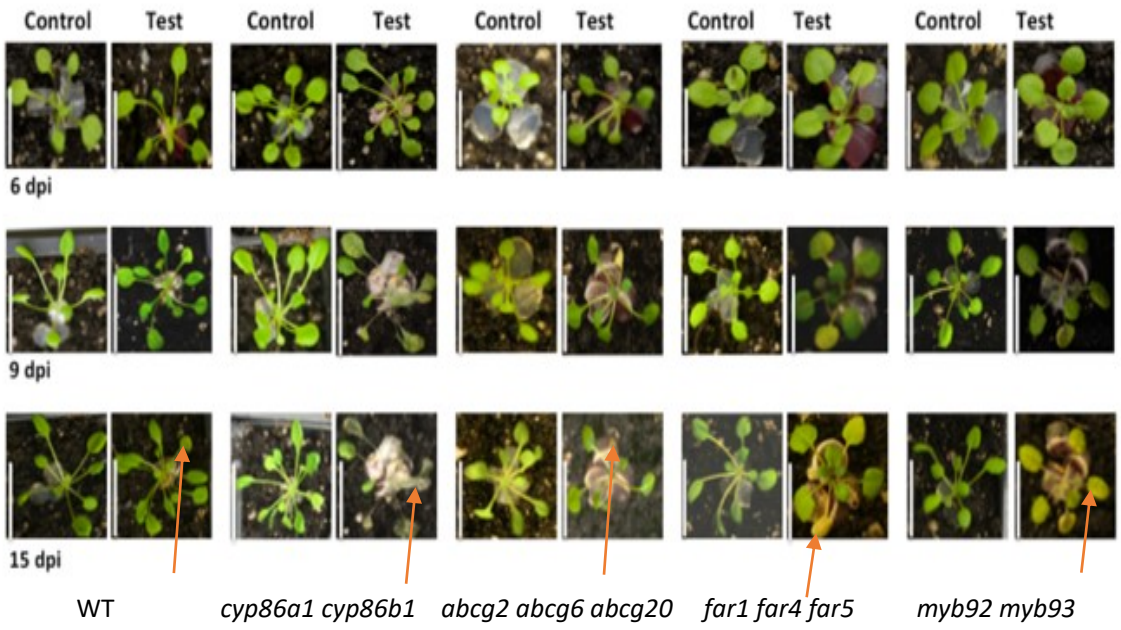
3.4.1 Effects of cell wall suberization in root endoderm and / or periderm on fungal pathogen susceptibility in roots of Arabidopsis.

The roots of four-week-old plants (twelve plants per genotype) grown in trays were challenged with four 6-mm diameter plugs from the colony margin of 5-6-day old cultures of *Fusarium avenaceum* by placing the plugs around the root. Similarly, inoculum plugs were placed below the roots of plants grown on 1% Murashige and Skoog (MS) medium plates. In addition, *Fusarium avenaceum* spores at the concentration of 10^8 spores ml^{-1} were used to inoculate the roots of plants grown directly on soil. Indications of plant disease were monitored daily for two weeks, and photographs were taken at indicated time intervals (Figure 3-1).

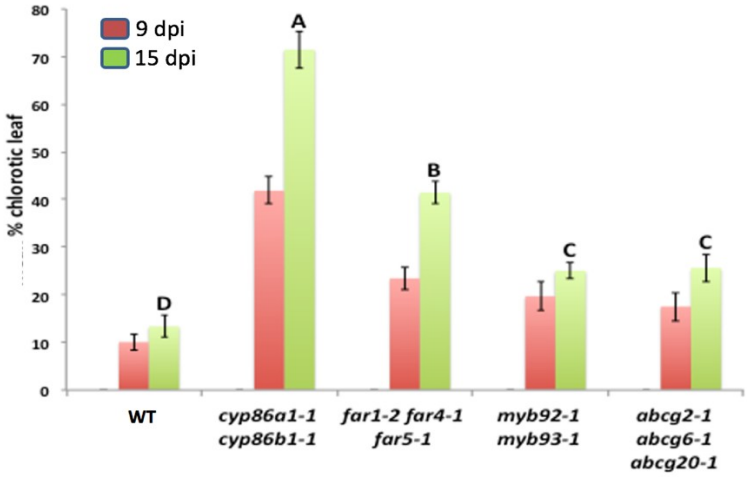
Leaf chlorosis and dryness was observed on the suberin mutants and wild type plants in planting trays that were challenged with plugs of *F. avenaceum*. Degree of leaf chlorosis and dryness was more prominent in the suberin mutants than in the wild type plants. For instance, ~70% of inoculated *cyp86a1-1 cyp86b1-1* leaves were chlorotic at 15 days post

infection. Similarly, about 40% of *far1-2 far4-1 far5-1* leaves were chlorotic while ~30% *myb92 myb93* and *abcg2-1 abcg6-1 abcg20-1* leaves were chlorotic, as compared with wild type where only ~ 12% of the inoculated leaves were chlorotic. However, the leaves of plants inoculated with spores of *F. avenaceum* showed no chlorosis or any disease symptoms. Also, plants grown on 1% Murashige and Skoog (MS) medium that were inoculated with plugs of *F. avenaceum* mycelium showed no sign of infection.

A



B



C

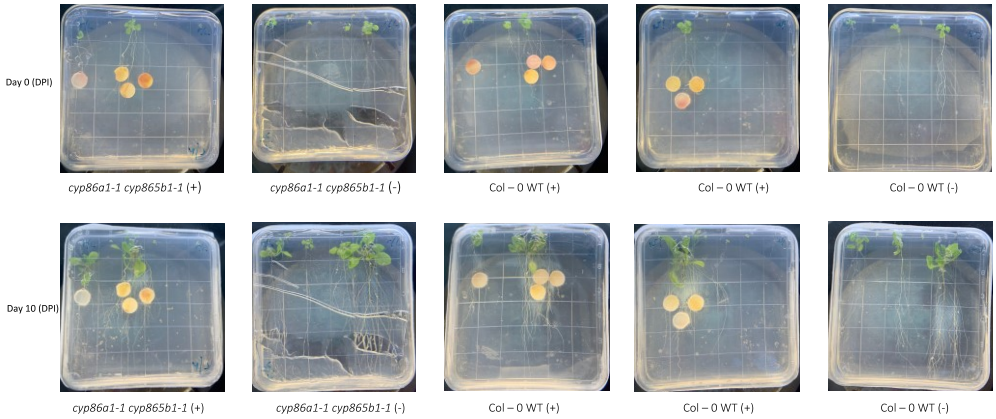


Figure 3-1. Response of suberin mutants *cyp86a1-1 cyp865b1-1, far1-2 far4-1 far5-1, myb92-1 myb93-1, abcg2-1 abcg6-1 abcg20-1* and wild type to *F. avenaceum*.

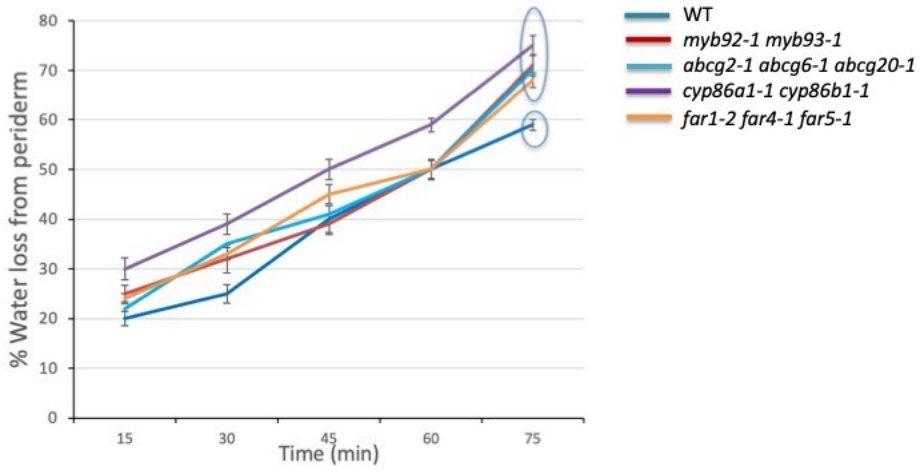
A) Leaf chlorosis and dryness of *cyp86a1-1 cyp865b1-1* in response to *F. avenaceum* at 6-, 9- and 15-days post infection (dpi) in root area in terms of % leaf chlorosis. Scale bar at left of each row is 1 cm. Arrows indicate chlorotic leaf. B) Percent of chlorotic leaves on 9- and 15-dpi for wild type and suberin mutant plants grown in soil and inoculated with *F. avenaceum*. Note that, in the most severe case, ~70% of *cyp86a1-1 cyp865b1-1* leaves were chlorotic at 15 dpi. Different letters indicate significant differences between genotypes at 15 dpi ($P < 0.05$, ANOVA, HSD post hoc, $\alpha = 0.05$). $N \geq 10$ for each genotype. C) Example of susceptibility assay of *cyp86a1-1 cyp865b1-1* and wild type Arabidopsis to *F. avenaceum* at 0- and 10-dpi to examine leaf chlorosis and root decay in MS medium plates. (+) inoculated, (-) uninoculated. No symptoms of either chlorotic leaf or root decay were observed in any of these treatments. Experiment was repeated three times.

3.4.1.1 Measurement of root periderm segments for water loss

The results shown in Figure 3-1A may be explained by increased susceptibility of suberin mutants to infection by *F. avenaceum* or by other associated phenotypes of the suberin mutants, such as desiccation tolerance, for example. I went further to examine if leaf chlorosis and dryness observed on the soil-grown plants with suberin defects was due to water loss via the plant roots. This phenotype was tested by measurement of root periderm segments for water loss with or without exposure to the fungus. I analysed the amount of water loss in mature root periderm segments of suberin mutants in comparison them with wild type. A 1-cm segment of tap root at the base of the root system was cut from 4-week-old plants. A thin layer of Vaseline was applied to the cut surfaces (to prevent water loss), and the weight was recorded promptly. The root segments were then placed on the laboratory bench and weights were recorded at 15 minutes intervals. The loss of fresh mass was calculated as percentage of the initial mass.

The results of this experiment showed that by the end of 75 minutes, the amount (%) of water loss by suberin mutants was noticeably greater than the water loss from the wild type for both *F. avenaceum* inoculated plants (Figure 3-2A) and mock treated plants (Figure 3-2B). In both cases, *cyp86a1-1 cyp86b1-1* lost the greatest amount of water compared to other mutants and in comparison, to wild type.

A



B

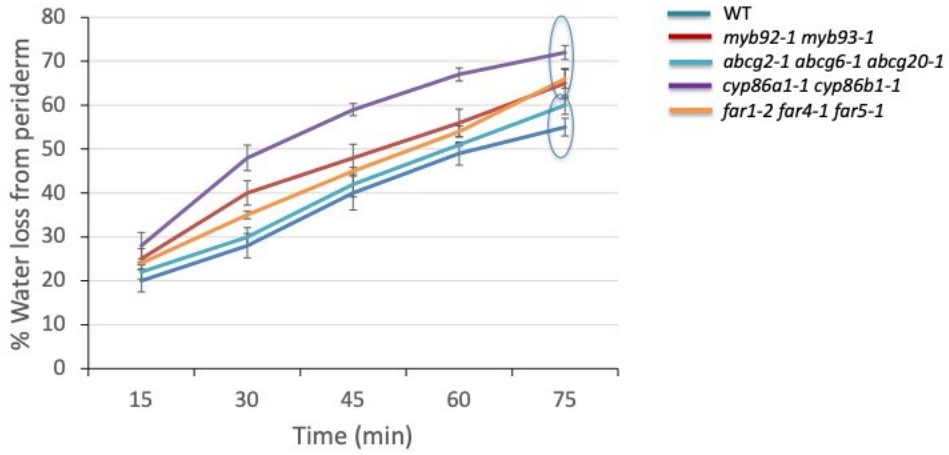


Figure 3-2. Amount of water loss through the root periderm segment of wild type, *cyp86a1-1 cyp86b1-1, myb92-1 myb93-1, far1-2 far4-1 far5-1* and *abcg2-1, abcg6-1 abcg20-1*plants.

Circles identify groups with significant differences at 75 minutes ($P < 0.01$, ANOVA, Tukey HSD post hoc, $\alpha=0.05$). Values represent mean ± SD, n=5, with each replicate containing 3 periderm segments from different plants. A) *F. avenaceum* inoculated plants. B) Mock-inoculated plants. Experiment was repeated three times with similar results.

3.4.1.2 Ectopic suberin expression in Arabidopsis leaves does not act as barrier to the invasion of leaf bacterial pathogen

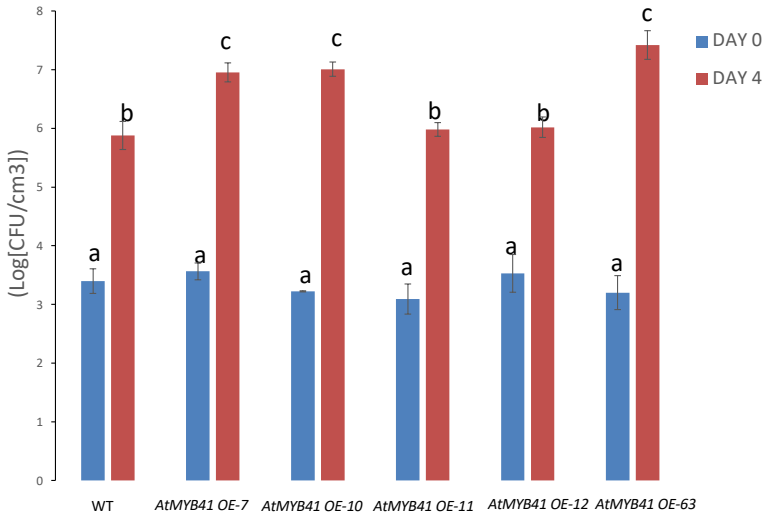
I investigated the susceptibility of mutant plants expressing suberin ectopically in leaf tissue to the pathogen *Pseudomonas syringae hrcC*$^{-}$. Susceptibility was measured by pathogen-treating the leaves of *AtMYB41 OE-7*, *AtMYB41 OE-10, AtMYB41 OE-11*, *AtMYB41 OE-12* and *AtMYB41 OE-63* mutant and wild type plants. The pathogen was cultivated, and spray-inoculated onto the plant leaves, and disease progression was subsequently monitored by bacterial colony counts.

The results of this experiment showed that ectopic suberin expression in Arabidopsis leaves does not prevent the invasion of the leaves by *P. syringae hrcC*$^{-}$. Unexpectedly, and contrary to my hypothesis, *AtMYB4 OE-7, AtMYB4 OE-10* and *AtMYB4 OE-63* harboured significantly greater numbers of bacteria than the wild type by day 4. *AtMYB41 OE-11* and *AtMYB41 OE-12* had equivalent numbers of bacteria as did the wild type control. Moreover, twelve days post infection, *AtMYB41 OE-7* and *AtMYB41 OE-63* - the mutants with highest levels of ectopic suberin in leaf tissues had yet to recover from the infection based on visual inspection of plant condition (Figure 3-3B). At 12 days post infection, wild type plants appear to be fully recovered from pathogen attack and *AtMYB41 OE-10, AtMYB41 OE-11,* and *AtMYB41 OE-12* were apparently recovering.

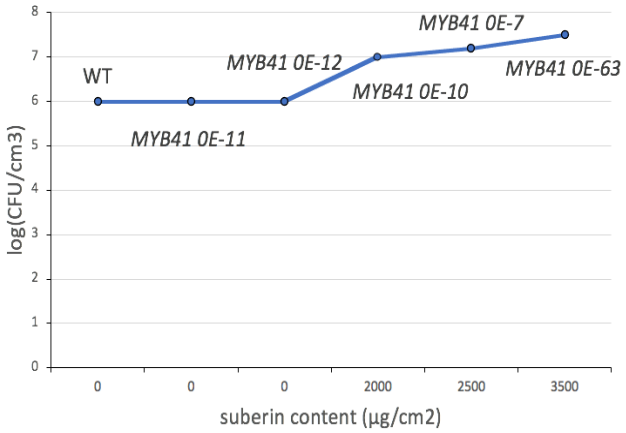
The results indicated, overall, that the wild type plants were more resistant to infection by *P. syringae hrcC*$^{-}$ compared to mutant plants that ectopically express suberin in leaf tissue. This conclusion is based on the premise that increased titres of *P. syringae* within a leaf is an indication of greater infection. That recovery from pathogen attack occurred more rapidly in wild-type plants, in general, than in suberin mutant plants could also indicate increased disease susceptibility by the suberin mutants. However, other

factors such as water stress may again be involved. Arguing for the later, the wild type plants may have a better capacity to retain water and nutrients than the mutant plants, and this could compound the effects of the pathogenic bacterium. This view is congruent with the capacity of leaves from the wild type plants to retain water more than suberin overexpression mutants, as shown by the analysis of leaf RWC (Figure 3-6, below).

A



B



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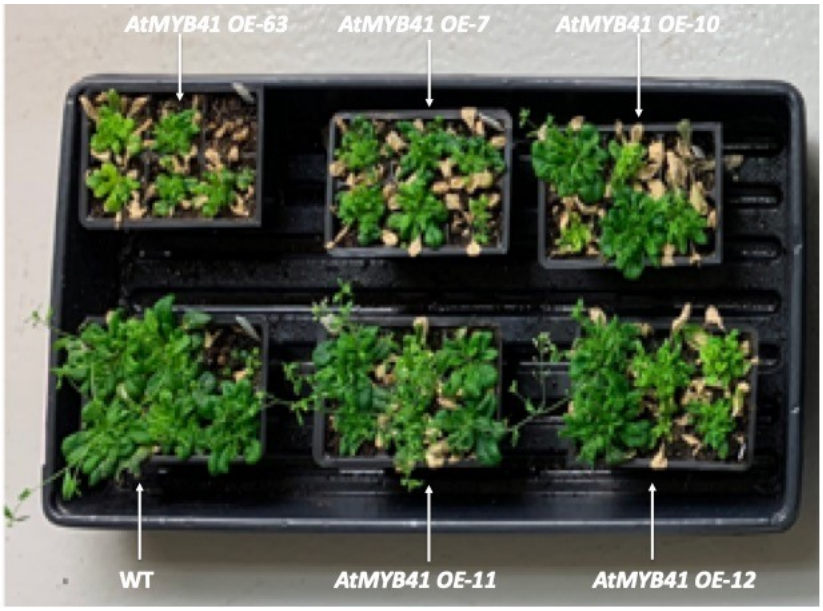


Figure 3-3. Susceptibility of *AtMYB41 OE* mutant plants and wild type plants to *Pst hrcC*$^-$.

A) Plants were sprayed with 10^8 cfu/ml of *Pst hrcC*$^-$ on day 0. Bacterial titres were monitored immediately after inoculation and on day 4. Means (+/- SD) are plotted based on three replicates, each replicate represents bacterial enumeration from 4 leaf discs from two plants. Different letters indicate significant differences between genotypes ($P < 0.05$, ANOVA, HSD post hoc, α=0.05). B) Positive correlation between suberin content and bacterial growth at 4 dpi. C) Twelve days after inoculation, *AtMYB41 OE-7* and *AtMYB41 OE-63*, in particular, are yet to recover from initial inoculations with *Pst hrcC*$^-$, compared to the wild type that has apparently fully recovered from pathogen exposure.

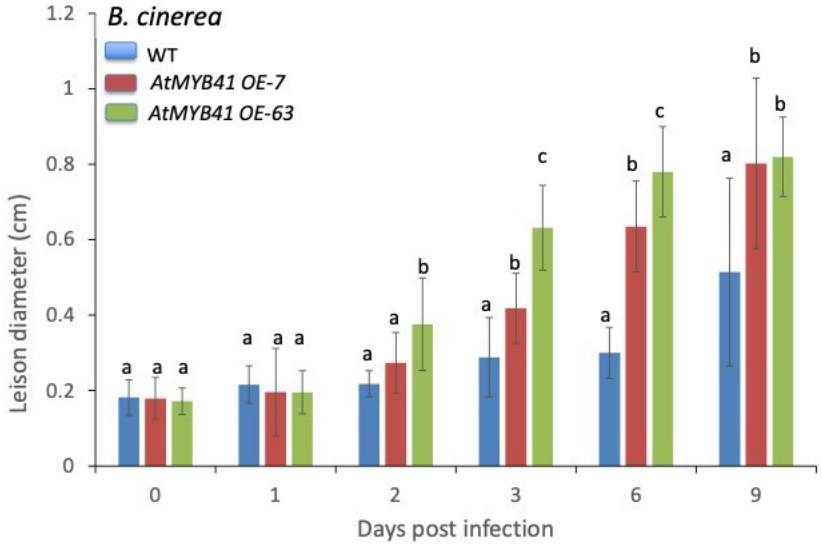
3.4.1.3 Ectopic suberin expression in Arabidopsis leaves does not act as barrier to the invasion of pathogenic fungus

I investigated the susceptibility of mutants ectopically overexpressing suberin in the leaves to the fungal pathogen *Botrytis cinerea*. This was done by direct pathogen tests on the incised leaves of wild type, *AtMYB41 OE-7* and *AtMYB41 OE-63.* The pathogen was cultivated to obtain conidia that were inoculated onto the plants leaves after which disease progression was monitored.

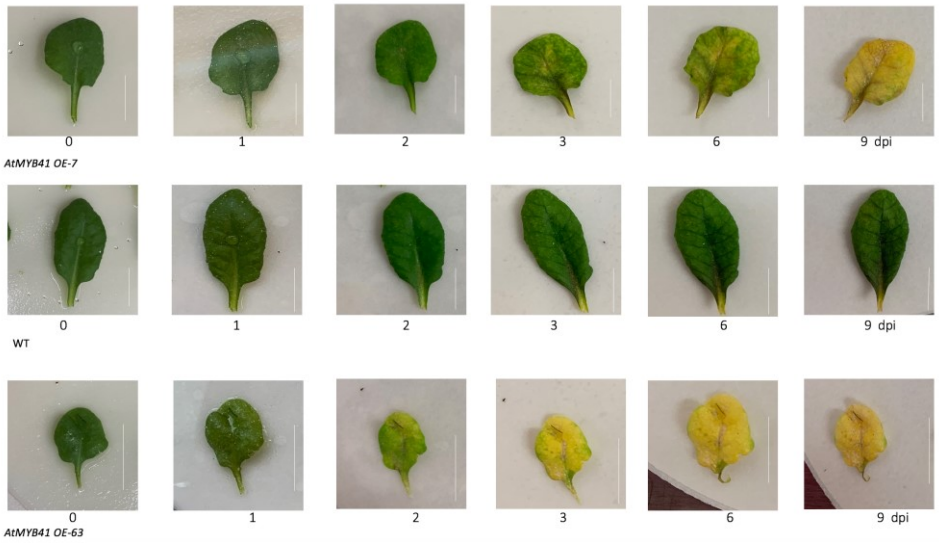
A


0
1
2
3
6
9 dpi
AtMYB41 OE-7
WT
AtMYB41 OE-63

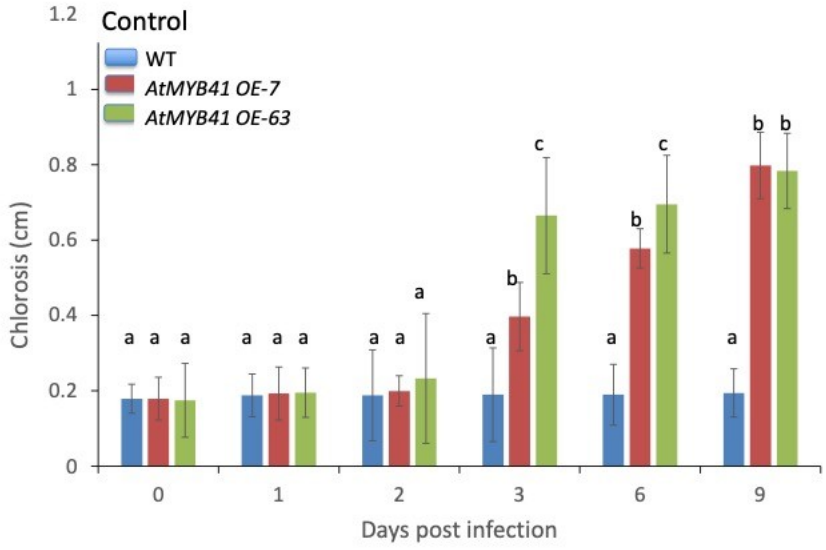
B


B. cinerea
WT
AtMYB41 OE-7
AtMYB41 OE-63
Leison diameter (cm)
Days post infection
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
6
9

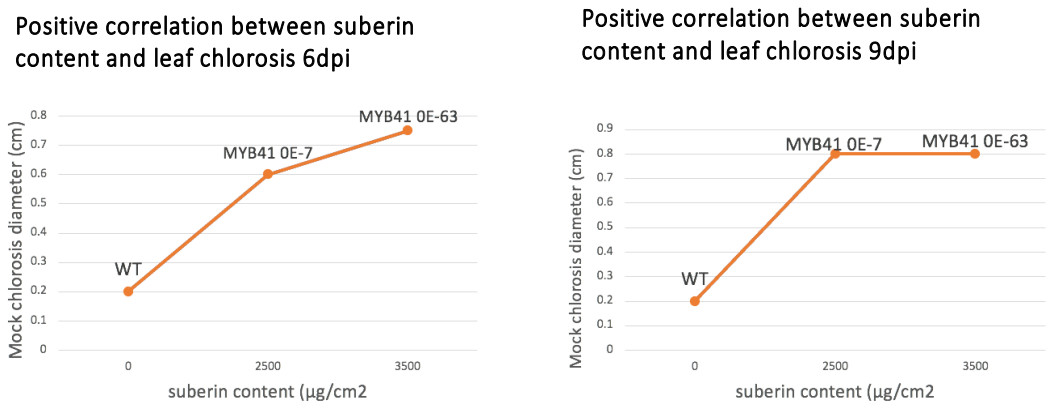
C


0
1
2
3
6
9 dpi
AtMYB41 OE-7
WT
AtMYB41 OE-63

D


Control
WT
AtMYB41 OE-7
AtMYB41 OE-63
Chlorosis (cm)
Days post infection
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
6
9
a a a a a a a a a a b c a b c a b b

E



F

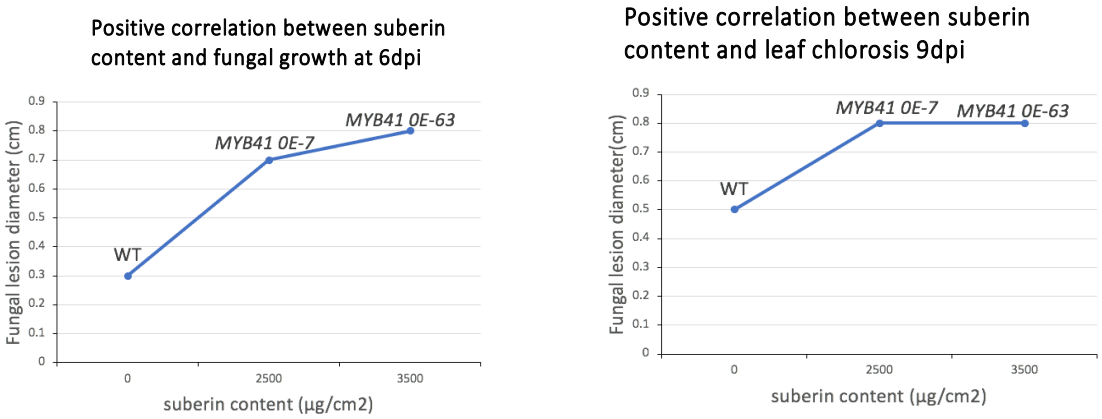


Figure 3-4. Susceptibility of *AtMYB41 OE-7* and *AtMYB41 OE-63 and* wild type plant leaves to *B. cinerea* at 0 to 9-days post infection

Susceptibility was measured in terms of diameter of pathogen lesion size. A) Image showing infection of *B. cinerea* on inoculated plant leaves. B) Lesion diameter of chlorotic leaves due to *B. cinerea* infection. n=12, with each replicate containing 1 leaf from different plants. Different letters indicate significant differences between mutants and wild type plants ($P < 0.01$, ANOVA, Tukey HSD post hoc, a=0.05). Values represent mean ± SD). C) Chlorosis of uninoculated leaves, D) Chlorosis of uninoculated leaves. n=12, with each replicate containing 1 leaf from different plants, E) Positive correlation between suberin content and chlorosis for mock treated plants, F) Positive correlation between suberin content and chlorosis for *Botrytis cinerea* infected plants. Different letters indicate significant differences between mutants and wild type plants ($P < 0.01$, ANOVA, Tukey HSD post hoc, α=0.05). Values represent mean ± SD). Experiment was repeated twice with similar results.

Results showed the leaves of both *B. cinerea*-infected and mock treated plants developed chlorotic and dry-tissue symptoms over time. Leaf chlorosis and dryness observed in the inoculated plants (Figure 3 - 4 A, B) may, therefore, be due to *B. cinerea* infection spread from the point of contact to other areas of the leaves and/or water loss from the epidermis. Chlorosis formation occurred earlier in *B. cinerea*-infected leaves of *AtMYB41 OE-7* and *AtMYB41 OE-63* (during days 1-2), whereas chlorosis begins to appear by the third day in the wild type leaves. The pathogen appeared to also grow and spread faster in *AtMYB41 OE-7* and *AtMYB41 OE-63* compared to the wild type. Moreover, by the third day post infection, the pathogen had covered nearly all of the leaves of *AtMYB41 OE-63* compared to *AtMYB41 OE-7* and the wild type (Figure 3 -4 A, B). Chlorosis formation occurred earlier in *B. cinerea*-infected leaves compared to mock treated leaves. However, that similar chlorosis symptoms occurred with the control (untreated) plants must be attributed only to water loss from the epidermis and is not pathogen related, since no pathogen was inoculated (Figure 3-4 C, D). Therefore, it is difficult to clearly differentiate between apparent increases in susceptibility to the pathogen in mutants that overexpress suberin in leaves and their apparent increased permeability. In this case, enhanced growth by the pathogen may simply reflect increased permeability and ready access to nutrients by the pathogen.

3.4.1.4 *AtMYB41-OE* ectopic suberin expression in Arabidopsis leaves aids loss of water through the leaf surfaces

The ambiguity of pathogen response and counterintuitive loss of water from suberin-overexpressing plants prompted me to do additional water loss assays on suberin

overexpressing mutants. For this, 5 different plants of *AtMYB41 OE* mutant lines and wild type were grown in the growth room for 4 weeks under long day (16 h light/8 h dark). About 0.5 g of mature leaves from these plants were detached and weighed immediately to determine the fresh weight (FM). The leaves were then left on the laboratory bench and weighed at fifteen minutes intervals for one and half hours. The loss of fresh weight was calculated on the basis of the initial weight of the detached leaves, and the percentage of water loss was calculated.

The results indicate that leaves of *AtMYB41 OE-7, AtMYB41 OE-10* and *AtMYB41 OE-63* had a more rapid water loss than the leaves of the wild type plant. In these mutants, at two hours after excision, more than 60% of leaf moisture was lost. Rates of water loss in *AtMYB41 OE-11* and *AtMYB41 OE-12* was not statistically different from that of the wild type plants which lost approximately 35% water within the same period of time (Figure 3-5). These data support the idea that in spite of overexpressing suberin in the leaves, the *AtMYB41 OE* mutant plants have an overall higher rate of water loss than the wild-type plants.

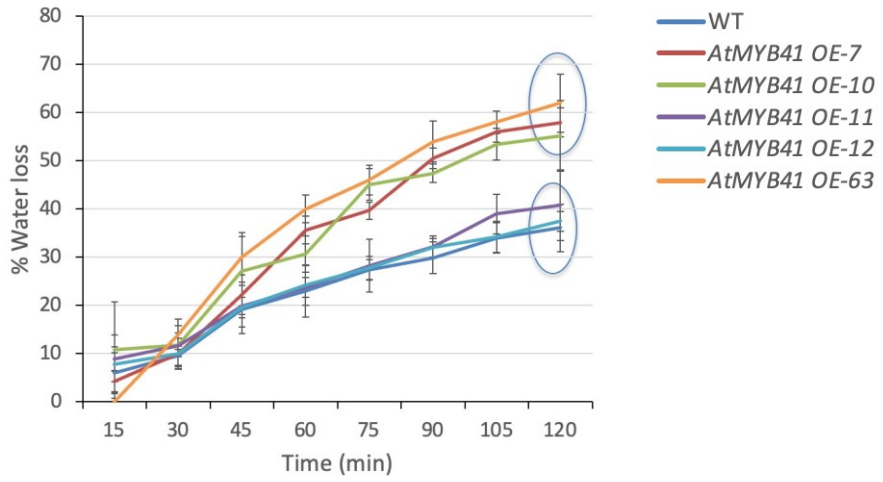


Figure 3-5. Percentage water loss of *AtMYB41 OE-7, AtMYB41 OE-10, AtMYB41 OE-11, AtMYB41 OE-12 and AtMYB41 OE-63* compared with wild type.

n=3, The plotted values are means +/- SD and circles identify groups with significant differences at 120 minutes ($P < 0.01$, ANOVA, Tukey HSD post hoc, $\alpha=0.05$). Experiment was repeated trice with similar results.

3.4.1.5 Ectopic suberin expression in Arabidopsis leaves does not increase the leaf relative water content (RWC)

Based on the observations of increased water loss through the leaf surfaces of *AtMYB41 OE-7, AtMYB41 OE-10 and AtMYB41 OE-63* compared with wild type, I proceeded to determine the relative water content (RWC) of the leaves from these genotypes. Leaf discs were collected from the fully developed leaves (one disc per leaf) of bacterial pathogen *Pst hrcC*$^{-}$ infected plants after four days of infection and control plants, to determine the leaf RWC. The leaf discs were weighed immediately to determine the fresh mass (FM). These leaves were then hydrated by floating in water for 4 hours at room temperature, after which the 'saturated mass' of the leaf discs (SM) were weighed after surface drying with paper towel. Leaves were then dried for 36 h at 60°C and reweighed

to determine the dry mass (DM). Leaf RWC % was calculated and is presented for each genotype in Figure 3-6. The state of water balance of a plant is represented by its leaf RWC, as this value expresses the total amount of water to reach artificial full saturation, that is, at full turgor (Pieczynski et al., 2013).

Results showed that ectopic suberin expression in Arabidopsis leaves of the mutants had decreased relative water content compared to wild type plant leaves. Without pathogen, the wild type has ~95% RWC, which is significantly greater than for the mutants - *AtMYB41 OE-7*, *AtMYB41 OE-10,* and *AtMYB41 OE-63*. The mutants *AtMYB41 OE-11* and *AtMYB41 OE-12* also have a lower RWC than wild type. This would suggest that the mutant plants are under water stress even when not infected with pathogen. In all cases, the uninoculated plants have higher % RWC than the pathogen infected plants. Furthermore, pathogen infection does not appear to disproportionately influence RWC in the mutant lines, based on the ratio of RWC (infected / RWC (mock).

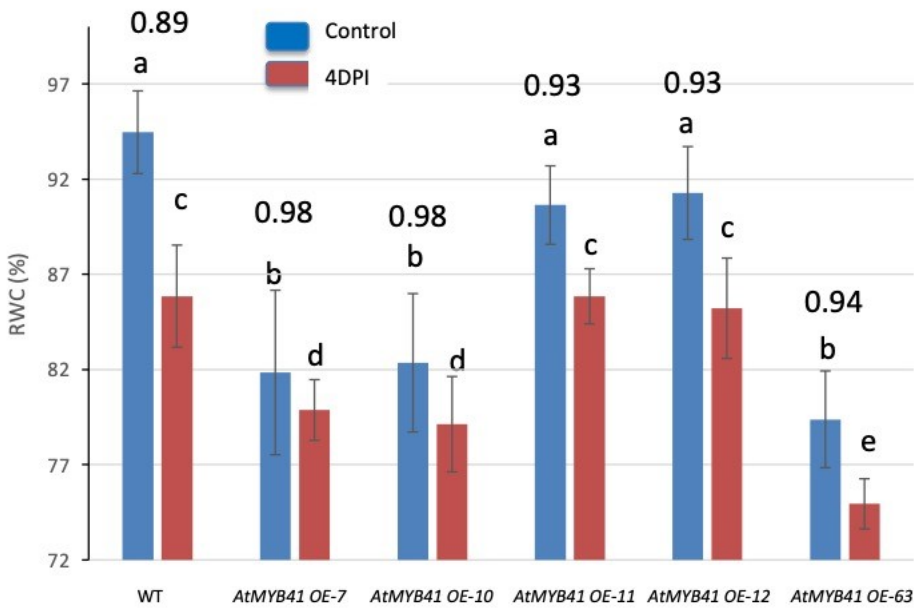


Figure 3-6. Percentage RWC of leaf disks of *AtMYB41 OE-7, AtMYB41 OE-10, AtMYB41 OE-11, AtMYB41 OE-12* and *AtMYB41 OE-63* compared with wild type with and without *Pst hrcC*⁻ inoculation.

Different letters indicate significant differences among plants ($P < 0.01$, ANOVA, Tukey HSD post hoc, $\alpha=0.05$). Numbers above each set of bars is the ratio of RWC (infected) / RWC (uninfected). Values are means +/- SD at 4 days post inoculation with *Pst hrcC*⁻ or the same day for uninoculated plants. Each replicate (n=6) contains 5 leaf disks from five plants. Experiment was repeated twice with similar results.

I. Chlorophyll leaching assay

A chlorophyll leaching assay was performed, and the results are presented in (Fig 3-7). This assay was used to further examine the permeability of the cuticle in mutant and wild type leaves. The chlorophyll leaching assay was performed using four-week-old plants from lines *AtMYB41 OE-7, AtMYB41 OE-10, AtMYB41 OE-11, AtMYB41 OE-12* and *AtMYB41 OE-63* and wild type grown under long days (16 h light/8 h dark) in soil. All of the genotypes had the same green colour appearance, which suggested normal chlorophyll content. For this assay, leaves from four-week-old plants were incubated in

tubes containing 80% ethanol in dark. A series of 1-mL aliquots were taken at specific time points and absorbance was measured at 647 and 664 nm to quantify the chlorophyll released into the solution. Micromolar concentrations of total chlorophyll per gram fresh weight in each of the samples were then calculated.

The results of this assay were congruent with the RWC trends (Fig 3-6) and showed that chlorophyll was generally more readily released from the mutants than from the wild-type leaves. Compared to wild type leaves, chlorophyll was released at significantly greater rates in the mutants *AtMYB41 OE-7*, *AtMYB41 OE-10,* and *AtMYB41 OE-63*, whereas mutants *AtMYB41 OE-11* and *AtMYB41 OE-12* showed slightly lower chlorophyll release trends similar to the wild type. Overall, these results suggest that the suberization of leaves in the overexpression mutants causes an increase in leaf permeability (Fig 3-7). We also infer from this assay that, in addition to water, nutrient release would be enhanced in the suberin overexpression mutants. This increased availability of water and nutrients is expected to increase microbial growth on the leaf surface and may facilitate infection.

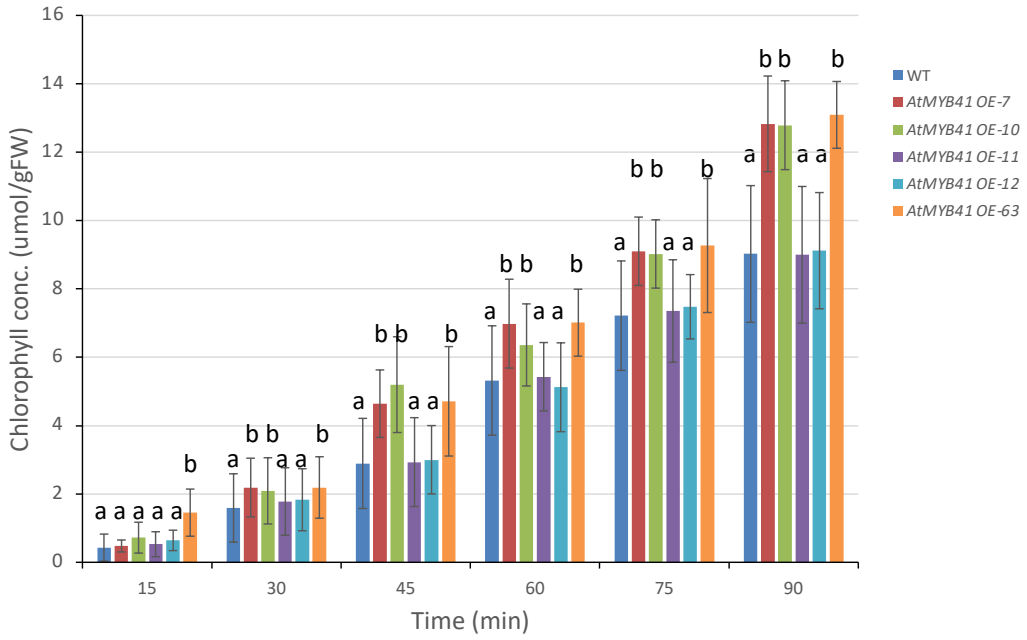


Figure 3-7. Chlorophyll-leaching values with mature leaves of suberin overexpressing mutants and wild type at designated times.

Different letters indicate significant differences between plant lines within each time point ($P < 0.01$, ANOVA, Tukey HSD post hoc, $\alpha=0.05$). Values are means +/- SD with n=3 for each bar. Experiment was done twice with similar results.

ii. Toluidine Blue O (TBO) assay

As a final test of leaf permeability, a Toluidine Blue O (TBO) staining assay was carried out with *AtMYB41 OE-7, AtMYB41 OE-11, AtMYB41 OE-63* and wild type. Leaves with a disrupted cuticle are expected to have intense blue staining because of fast infiltration of the stain into the leaf, whereas leaves with an intact cuticle should have relatively low stain uptake. Leaves were submerged in an aqueous solution of 0.05% (w/v) TBO and then excess stain was gently washed off with distilled water and leaves were photographed. The results of this assay are non-quantitative, but qualitatively support previous permeability assays in that the mutants *AtMYB41 OE-7* and *AtMYB41 OE-63* were

stained more rapidly with TBO than *AtMYB41 OE-11* and wild type, which were not appreciably stained. (Fig 3-8).

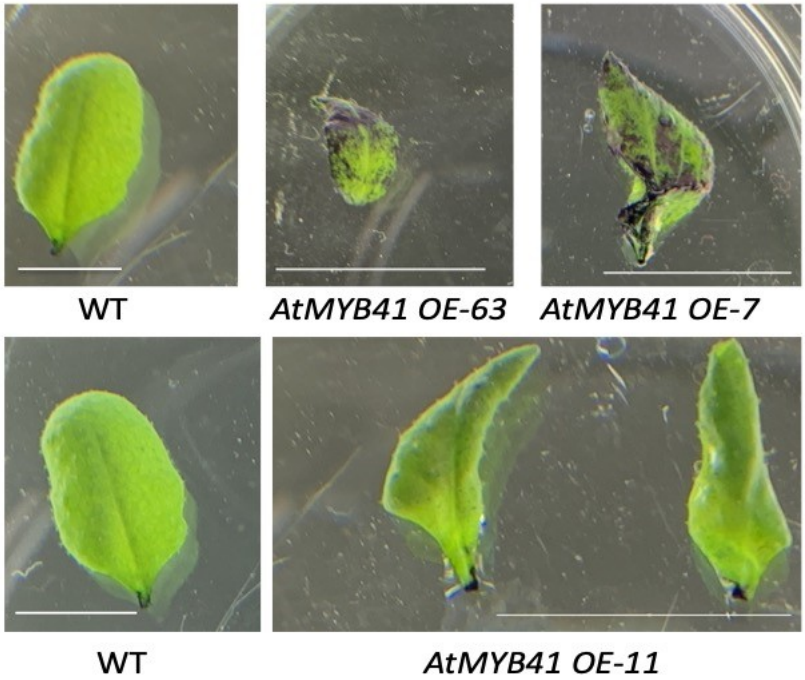


Figure 3-8. Figure 3-8. Toluidine Blue O staining of *AtMYB41 OE-7, AtMYB41 OE-11, AtMYB41 OE-63* and wild type.

Leaves from four-week-old plants immersed in TBO solution. Picture taken after 3-minute exposure to TBO stain Six leaves from three plants were analyzed per genotype. (Scale bar = 1 cm).

3.5 Discussion

3.5.1 Cell wall suberization in root periderm acts as barrier to water loss.

Under normal growth states, suberin is deposited in the root endodermis (primary young root) and periderm (root that has undergone secondary growth), leaf bundle sheath, seed coats and tubers (when present) (Mahmood et al., 2019; Lukas Schreiber, 2010). Suberin deposition in the root is believed to be further enhanced by abiotic and biotic

stresses with the main function of regulating the movement of water and solutes across the cell or tissue layers and also to impede pathogens from gaining entrance into tissues (Graça, 2015; Mahmood et al., 2019). Using the model plant *A. thaliana*, I investigated the resistance or susceptibility of mutants deficient in suberin monomers to a root fungal pathogen – *F. avenaceum.* I also investigated the effects of suberin deficiency on water loss via the root peridermis. This study entailed culturing of the pathogen, development of suitable pathogen assays by infecting plants with fungal pathogen strains, and monitoring disease symptoms in Arabidopsis wild type and suberin mutants. I evaluated disease severity based on plant morphology, size, growth restriction, and chlorosis in comparison to control plants.

The results show that mutants defective in suberin may have increased susceptibility to infection by *F. avenaceum*. For example, leaf chlorosis and dryness of *cyp86a1-1 cyp865b1-1* in response to *Fusarium avenaceum* at 15-days post infection (dpi) was striking. This is evident in that ~70% of inoculated *cyp86a1-1 cyp865b1-1* leaves were chlorotic at 15 days post infection. Similarly, about 40% of *far1-2 far4-1 far5-1* leaves were chlorotic while ~30% *myb92 myb93* and *abcg2-1 abcg6-1 abcg20-1* leaves were chlorotic, as compared with wild type where only ~ 12% of the inoculated leaves were chlorotic. This suggests that the roots of the suberin mutants tested are more susceptible to infection by this fungal pathogen than wild type.

However, it was also shown that these same suberin defects result in decreased barrier function against water loss in roots. Specifically, analysis of water content in roots confirmed that suberin defects resulted in increased water loss through the root periderm. For both *F. avenaceum* inoculated and uninoculated plants, all the suberin mutants tested

showed increased water loss through the periderm compared to the wild type. In particular, compared to the wild-type and other suberin mutants, *cyp86a1-1 cyp86b1-1* exhibited the most pronounced water loss via the root periderm ~74%. It was reported that the *cyp86a1-1 cyp86b1-1* double mutant has overall reduced suberin content (~60% of wild type) and has deformed lamellae structure due to reductions of C22- and C24-hydroxyacids and α, ω-dicarboxylic acids in root aliphatic polyesters (Compagnon et al., 2009). This result indicates that suberin deficiency associated with the double mutant, *cyp86a1-1 cyp86b1-1,* causes increased water loss through the root periderm. According to Li et al. (2017), a low rate of water loss is crucial for balanced metabolic processes and optimal water use efficiency. It is therefore possible that the enhanced 'disease symptoms' (chlorosis and leaf dryness) observed in suberin mutants (Fig 3-1A) was a result of rapid water loss via the root and/or due to the activities of the fungal pathogen. For example, loss of nutrients through root periderm may serve as food and enhance fungal growth in the root zone.

In addition, compared to wild type, *far1-2 far4-1 far5-1* which has ~70% reduction in total fatty alcohol in root, lost significantly greater water of approximately 70% across the root periderm of *F. avenaceum* inoculated plants. Similarly, *myb92-1 myb93-1* with reduction in total root suberin and *abcg2-1 abcg6-1 abcg20-1* with alterations in the suberin lamellae, composition and properties of root lost equal percentages (70%) of water. However, for mock treated plants, wild type and *abcg2-1 abcg6-1 abcg20-1* experienced a lesser water loss of approximately 60% compared to *cyp86a1-1 cyp86b1-1, far1-2 far4-1 far5-1, myb92-1 myb93-1* and *myb92-1 myb93-1* that lost approximately 70% water.

These results show that all of the suberin mutants tested are compromised for water retention in the root. However, these water balance effects obscure our interpretation of

pathogen susceptibility. In particular, it is possible that apparent increases in susceptibility of suberin mutants to the root pathogen, *F. avenaceum*, may be a side effect of increased permeability of the plant root providing greater access to nutrients in the root zones of soil-grown plants. Suberin deficiencies, in this case, would have an indirect influence on susceptibility.

3.5.2 Ectopic suberin expression in Arabidopsis leaves does not act as barrier to the invasion of leaf bacterial pathogen

As stated by Riederer & Schreiber (2001), the major barricade against unrestrained water loss from leaves, fruits, and other aerial parts of higher plants is the cuticle, which is viewed as a major barrier in protection against biotic and abiotic stresses (Cominelli et al., 2008). Based on this idea, I hypothesized that overexpression of suberin in leaves would result in reduced susceptibility to pathogen attack and a reduction in overall leaf permeability. Contrary to these predictions, my results show that ectopic expression of suberin did neither. Compared to wild type plants, I observed greater or equivalent susceptibility of leaves to the bacterial pathogen, *P. syringae Pst hrcC*$^{-}$ and to the fungal pathogen, *Botrytis cinerea*. This result was evident by the enhanced proliferation of *Pst hrcC*$^{-}$ in the leaves of *AtMYB41 OE-7*, *AtMYB41 OE-10 and AtMYB41 OE-63,* which have elevated leaf suberin, compared to the wild type (Figure 3-3A). The leaves of *AtMYB41 OE-11* and *AtMYB41 OE-12,* which are similar to wild type in having low suberin content in the leaves, yielded the same bacterial titres by 4 dpi as the wild type. Moreover, twelve days after inoculation, *AtMYB41 OE-7* and *AtMYB41 OE-63,* in particular, are yet to

recover from initial inoculations with *Pst hrcC*$^{-}$, compared to the wild type that has apparently fully recovered from pathogen exposure. (Figure 3-3B).

Results of infection with fungal pathogen showed that the leaves of both *B. cinerea* infected and uninfected control plants displayed chlorotic and dryness symptoms. Contrary to my hypothesis, lesions originating from the point of *B. cinerea* inoculation spread faster in overexpression mutants *AtMYB41 OE-7* and *AtMYB41 OE-63,* compared to the wild type. Moreover, by the third day post infection, the pathogen had covered nearly all of the leaves of *AtMYB41 OE-63* compared to *AtMYB41 OE-7* and the wild type (Figure 3-4B). However, the chlorotic and dryness symptoms obtained in *AtMYB41 OE-7* and *AtMYB41 OE-63* control (untreated) plants, once again, obscures the relationship of the mutations and pathogen susceptibility (Figure 3-4 C, D) and it is significant to note that uninoculated wild type leaves showed dryness symptoms but no chlorotic symptom in this assay. A predisposition to leakage of cell contents and resultant chlorosis by the plant, therefore, may enhance growth of a necrotrophic fungal pathogen such as *B. cinerea*. This makes it unclear whether or not suberin acts as a chemical inhibitor of fungal growth or may simply passively influence access to nutrients on leaves.

To further characterize perturbation of the leaf epidermis of overexpression mutants, four different permeability assays were done, based on water loss, relative water content, chlorophyll leaching, and Toluidine Blue O staining. The first of these two assays measure evaporation of water from the leaf surface and water content in leaves relative to a saturated state, respectively. The latter two assays evaluate whether or not an endogenous, large biomolecule (chlorophyll) and a positively charged ionic dye, respectively, can readily move across the plant cell membrane. These assays were remarkably congruent in

indicating that leaves of *AtMYB41 OE-7, AtMYB41 OE-10* and *AtMYB41 OE-63,* in particular, have greater overall permeability than leaves of the wild-type plant and other overexpression mutants. Based on results of other studies, my observations suggest that ectopic expression of suberin in plant leaves may, in some cases, interfere with the biosynthesis or deposition of the leaf cuticle. Notable is that similar phenotypes are observed with transgenic lines affected in cuticle biosynthesis which led to increased rate of rapid water loss compared to wild-type plants. Defects in cuticle biosynthesis appear to enhance permeability of leaf surfaces, suggesting disjointedness in the cuticle or cuticle defects (Cominelli et al., 2008). Furthermore, in a separate study by Mahmood et al., (2019), it was revealed that overexpression of the NAC (NO APICAL MERISTEM/ATAF1/CUP-SHAPED COTYLEDON2) transcription factor gene *ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN46* resulted in an increased expression of suberin biosynthesis genes in the leaves, which resulted in elevated fatty acids, specifically very-long chain-fatty acids (VLCFA) of C24 and C26, compared to the wild type. These overexpression lines exhibited defective leaf surfaces with increased permeability, as evident by rapid TBO stain uptake and increased chlorophyll leaching rates (Mahmood et al., 2019). NAC transcription factors have been shown to involved in plant responses to abiotic stress (Oda-Yamamizo et al., 2016). All of these data suggest that ectopic expression of suberin in plant leaves probably affects the integrity of the cuticle.

In this study, it was shown that although periderm suberization tends to reduce Arabidopsis root susceptibility to infection by *F. avenaceum*, and also prevent rapid loss of water from the peridermis of plant root; overexpression of suberin in leaves neither reduces permeability or susceptibility to pathogens *P. syringae* and *B. cinerea*.

CHAPTER 4: Summary and Conclusion

Plants occupy their natural environment in close association with diverse microorganisms. Many of these microorganisms are detrimental to the growth and success of plants, because they cause disease (Gupta et al., 2017). As early as the 19th century, experiments by Heinrich Anton de Bary showed that pathogenic microorganisms are the causative agents of plant diseases (Kutschera, U., 2012). Plants, as primary producers and the major source of food for humanity, need to thrive, be healthy, and be free of diseases (Abdul Malik et al., 2020; Allard & Micallef, 2019). As the world population increases, the emergence of new diseases is a global threat to food security (McCann, 2020). Scientists and plant pathologists are constantly seeking solutions that will improve food productivity and food security through crop breeding techniques aimed at producing resistance to biotic and abiotic stresses.

Arguably, plant defense responses are key to crop plant survival and food security, and understanding pathogen resistance and plant immunity will be beneficial to agricultural production by reducing crop loss, and also contribute to the understanding of the molecular interactions and coevolution that underlies other biological systems (Andersen et al., 2018). Defense responses are energy consuming and often come at the expense of plant growth. To detect and ward off pathogenic microorganisms, plants use a highly effective and dynamic defense strategy including physical and chemical barriers, as well as both innate and induced immunity (Abdul Malik et al., 2020; Jones & Dangl, 2006). My research investigates a form of physical/chemical barrier and a form of induced immunity, and there may be interactions in these general classes of defense.

The purpose of the second chapter of my thesis was to explore possible roles for Arabidopsis BOPs in induced plant defense using pathogen assays. BOPs are BTB-ankyrin

transcription co-factors that are evolutionarily conserved in plants. In mosses and higher plants, BTB-ankrin proteins are split into NPR1-like and BOP-like subgroups. In flowering plants, NPR1 perceives salicylic acid to activate SAR and BOPs regulate plant development. A role for BOP1/2 in plant defense has long been speculated based on structural similarity to NPR1, but direct evidence has been lacking (Hepworth et al., 2005; Wang et al., 2019). Canet et al. (2012) showed that the *bop1 bop2* mutant was defective in a priming response to methyl jasmonate suggesting a possible role in JA-mediated plant defenses. A microarray study followed by GO analysis of up-regulated genes in *BOP1-oe* plants revealed the promotion of genes associated with biotic stress (Khan et al., 2015). These observations prompted my use of *P. syringae* pv. *tomato* (*Pst*) DC3000, *Pst hrcC*$^{-}$, and *B. cinerea* to study the involvement of Arabidopsis BOPs in defense against pathogens.

The purpose of the third chapter of my thesis was to investigate if Arabidopsis root suberization, a form of physical/chemical barrier, prevented invasion by root pathogenic fungus *F. avenaceum*, and if ectopic expression of suberin in leaves prevents invasion by the leaf bacterial and fungal pathogens *P. syringae* and *B. cinerea*, respectively. I also examined water loss dynamics in suberin mutants with or without pathogen. Suberin is a hydrophobic heteropolymer deposited in the cell walls of various internal and external tissues in plants where it plays multiple roles at plant-environment interfaces (Franke & Schreiber, 2007; Graça, 2015; Kosma et al., 2014). An example of such a function is forming a barrier against uncontrolled water and solute movement in the root (Baxter et al., 2009). All land plants deposit suberin in root endodermis and in periderm tissues. The primary root contains endodermis and the outer covering of the above-ground and below-ground tissues that undergo secondary development form a periderm (Wunderling et al.,

2018). Despite the many speculations about suberin acting as a barrier to resist pathogen invasion in plant, there has been no evidence of direct pathogen test on root suberin (Churchward et al., 2018; Correia et al., 2020; Lulai & Corsini, 1998; Thomas et al., 2007; Vishwanath et al., 2013).

I summarized my findings on chapters two and three of my study below.

4.1 *BLADE-ON-PETIOLE* genes confer resistance to diverse pathogens

According to van Wersch et al. (2016), plants that constitutively activate defense genes often show resistance to diverse pathogens. In the first part of Chapter 2, I analyzed the susceptibility or resistance of *BOP* mutants to the hemi-biotrophic bacterium *P. syringae* pv. *tomato* (*Pst*) DC3000, which elicits a strong SA-dependent defense response (Kim et al., 2008) and *B. cinerea,* a necrotrophic fungal pathogen that elicits a strongly JA/ET-dependent defense response (Windram et al., 2012).

4.1.1 Summary and findings on pathogen resistance of *BOP* mutants

In my study, and as I hypothesized, plants overexpressing *BOP1 (BOP1-oe)* showed heightened resistance to both *P. syringae* pv. *tomato* (*Pst*) DC3000 and *B. cinerea,* whereas for both bacterial and fungal pathogens, *bop1 bop2* plants were susceptible to compared to wild-type plants. The susceptibility of *bop1 bop2* plants to *P. syringae* was evidenced by proliferation of the bacterial pathogen in the leaves of the plant, unlike *BOP1-oe* plant leaves that yielded significantly lower titres of the pathogen (Figure 2.1). Susceptibility to *B. cinerea* was evidenced in *bop1 bop2* by significantly greater spread of the lesion diameter compared to wild type, whereas lesion diameter in *BOP1-oe* leaves was reduced

compared to the wild type (Figure 2.2). With these outcomes, my hypothesis and predictions were supported; specifically, *BOP* genes have a broad-spectrum role in plant defense, plants that overexpress BOP1 or BOP2 will have increased resistance to both fungal and bacterial pathogens, and plants that are defective in BOP1/2 activity will be less resistant than wild type to infection with fungal and bacterial pathogens.

It is generally known that resistance to biotrophic pathogens is facilitated through SA signaling, and resistance to necrotrophic pathogens is mediated through JA signaling (Glazebrook, 2005). According to Rowe & Kliebenstein (2008) , plants that are impaired in JA signaling are more susceptible to necrotrophic fungal pathogens, such as *B. cinerea*. It is known that once in contact with plants, biotrophic pathogens first infect the epidermal cells, and then develop haustoria to contact the plant cells to uptake the nutrients from the living cells, whereas necrotrophic pathogens kill plant cells first, and then feed on the dead tissues (Zhang et al., 2018). For instance, *B. cinerea* is known to kill hosts using microRNAs to hijack the host RNA interference machinery to enhance virulence (Weiberg et al., 2013). Hemi-biotrophic pathogens like *P. syringae* will first use a biotrophic lifestyle and then enter a necrotrophic mode (Block & Alfano, 2011). Canet et al. (2012) described a possible role for BOPs in JA-mediated defense when they observed that *bop1 bop2* was defective in pathogen resistance induced by exogenous methyl jasmonate. Also, Castelló et al. (2018) demonstrated that NPR1 and BOP1/2 have the capacity to bind SA *in vitro*. Moreover, GO analysis of up-regulated genes in *BOP1-oe* plants resolved by microarray showed a large fraction of genes related to biotic stress with genes from SA and JA/ET defense pathways represented (Khan et al., 2015). With the results of my study showing that BOPs promote resistance to bacterial and fungal pathogens with diverse lifestyles, it

is therefore conceivable that BOP1/2 play a crucial role in plant defense.

4.2 *BOP* genes are required for PTI and potentially function in the same pathway as TGA1 and TGA4

GO analysis of up-regulated genes in *BOP1-oe* plants resolved by microarray showed a large fraction of genes related to biotic stress, with pattern-triggered innate immunity (PTI) related genes more abundant, in particular (Wang et al., 2020). This implies that BOPs are involved in this branch of plant immunity. Therefore, in the second part of Chapter 2, I analyzed the susceptibility or resistance of *BOP* and *TGA* mutants to the *hrcC*$^{-}$ mutant of *P. syringae (Pst hrcC*$^{-}$*)* that is able to mount only PTI defense response, because it is defective in type III secretion (Hauck et al., 2003).

4.2.1 Summary and findings on BOP role in PTI response

My study showed that *BOP1-oe* plants showed enhanced resistance to *Pst hrcC*$^{-}$ whereas *bop1 bop2, tga1 tga4,* and *bop1 bop2 tga1 tga4* plants displayed a small but significant increase in susceptibility compared to wild type plants. The resistance of *BOP1-oe* was associated with reduced bacterial titres *Pst hrcC*$^{-}$*,* and higher levels of ROS and callose deposition upon elicitation with *Pst hrcC*$^{-}$ and flg22 compared to the wild type. ROS burst is an early defense response triggered upon pathogen perception or the addition of an elicitor (Boller & Felix, 2009). This response was significantly enhanced in *BOP1-oe* plants and diminished in *tga1 tga4* and *bop1 bop2 tga1 tga4* mutants compared to wild-type. ROS production in wild type and *bop1 bop2* plants look similar. Further, pathogen-

induced callose deposition was significantly enhanced in *BOP1-oe* and diminished in *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* mutants. These findings clearly support my hypothesis that *BOP* genes are involved in PTI and function in the same pathway as clade I TGAs. Conversely, plants that are defective in BOP1/2 activity are predicted to show reduced PTI defense responses.

4.3 Future direction

My findings showed that BOP1/2 promote resistance to bacterial and fungal pathogens with diverse lifestyles and contribute to plant innate immunity. This finding provides concrete evidence that BTB-ankyrin proteins from BOP subclades contribute to plant defense. Future work will investigate if plants overexpressing BOP2 (*BOP2-oe*) has similar responses as *BOP1-oe*. Also, *BOP1-oe tga1 tga4 triple* mutant can be used to test whether the constitutive defense response in *BOP1-Oe* is suppressed by the *tga1 tga4* mutations. Additional bacterial pathogens such as the virulent strain, *P. syringae* pv maculicola ES4326 (*Psm)* could be used for these studies. Also, in the future, it will be interesting to study the mechanisms, particularly the possible interactions between BOP1/2 and NPR1, to control plant immunity. Furthermore, questions on interactions between plant defense, development, and morphology are of interest, since *BOP* genes were initially characterized for their role in development and maintenance of organ boundaries. Understanding these interactions will provide more knowledge on how to improve crop productivity along with disease resistance in crop plants. There is also the need to develop modified pathogen assays, such as inoculating attached leaves with agar blocks or impregnated filter paper disks containing fungi such as *Botrytis cinerea*.

4.4 Cell wall suberization in root periderm acts as barrier to water loss.

Roots are important organs for the survival of plants. Roots anchor plants firmly into the soil and are also forage for nutrients in the soil (Barberon, 2017; Bengough et al., 2016). The root and shoot system of plants differ greatly in defense strategies against pathogenic microorganisms. Some recognised gene markers for defense responses usually employed in leaves are less significant in roots and may not even be expressed (Chuberre et al., 2018). Most knowledge about plant immunity is based on studies with the aerial part of the plant, but according to Chuberre et al. (2018), the root system is the part of plant that confronts the highest number, variety and diversity of microorganisms. Infection of the plant root has a negative effect on crop production, but to date, few studies are dedicated to root defenses, as root systems are more complex to study because of their inaccessibility. In addition, soil-borne microorganisms are not easily culturable *in vitro* compared to leaf pathogens. It is known that less than 1% of soil microorganisms have been described and are culturable in the laboratory (Chuberre et al., 2018). The cell wall of plant root epidermis is the first barrier against pathogen attack, and suberization in cell walls may be useful in structural resistance. In the first part of chapter 3, I analyzed the susceptibility or resistance of mutants of suberin with varying root suberin content to *F. avenaceum* and also examined the rate of water loss through the roots of Arabidopsis mutants and wild type with or without pathogen inoculation.

4.4.1 Summary and findings on disruption of suberin in roots

My study provides evidence that suberin deposition in plant roots contributes to prevention of pathogen invasion of Arabidopsis, in that mutants tested showed chlorotic leaf and dryness compared to the wild type. For example, leaf chlorosis and dryness of *cyp86a1-1 cyp865b1-1* in response to *Fusarium avenaceum* was striking; ~70% of inoculated *cyp86a1-1 cyp865b1-1* leaves were chlorotic at 15 days post infection. CYP86A1 encodes a cytochrome P450 fatty acid ω-hydroxylase, which is a key enzyme for suberin biosynthesis**,** specifically in biosynthesis of aliphatic root suberin in Arabidopsis (Höfer et al., 2008). These observations support my hypothesis that cell wall suberization acts as barrier to the invasion of plant root by fungal pathogens. This finding is congruent with results of a recent study that showed that suberin accumulation in cell walls plays a role in defense against the invasion of *Verticillium dahliae* in wilt-resistant sea-island cotton plant root. The cotton homolog of CYP86A1 (GbCYP86A1), specifically expressed in roots and induced by *V. dahliae,* contributed significantly to resistance. When the gene was silenced, the plant had severely compromised resistance to *V. dahliae,* while heterologous overexpression of *GbCYP86A1-1* in Arabidopsis improved tolerance (Wang et al., 2020).

I also found evidence that suberin is involved in water movement regulation in and out of the plant system, since mutants deficient is root suberin lost more water via the periderm compared to wild type Arabidopsis plants. This observation complicates the issue since it is difficult to differentiate between the effects of suberin as a barrier to pathogen invasion, and as a barrier to water loss. Analysis of water content of roots confirmed that suberin defects resulted in increased water loss through the root periderm for both *F.*

avenaceum inoculated and uninoculated plants. In fact, all the suberin mutants tested showed increased water loss through the periderm compared to the wild type. Notably, *cyp86a1-1 cyp86b1-1* plants inoculated with *F. avenaceum* exhibited the most pronounced water loss via the root periderm at ~74%, compared to the wild type and other suberin mutants. The *cyp86a1-1 cyp86b1-1* double mutant has overall reduced suberin content (~60% of wild type) and has deformed lamellae structure due to reductions of C22- and C24-hydroxyacids and α, ω-dicarboxylic acids in root and seed coat aliphatic polyesters (Compagnon et al., 2009). Nevertheless, the question remains on how to differentiate between the effects of fungal invasion and water loss effects using this model.

4.5 Ectopic suberin expression in Arabidopsis leaves does not act as barrier to the invasion of leaf bacterial pathogen

In the second part of Chapter 3 of my thesis, I investigated if ectopic expression of suberin in Arabidopsis leaves prevent invasion by bacterial (*P. syringae*) and fungal (*B. cinerea*) pathogens. My study is the first to examine pathogenesis in *35S* promoter-driven overexpression of *AtMYB41* and the resulting ectopic accumulation of suberin in leaves of Arabidopsis (Cominelli et al., 2008; Kosma et al., 2014). It was previously established that *35S* promoter-driven overexpression of *AtMYB41* (*AtMYB41 OE-9*) leads to accumulation of suberin monomers in Arabidopsis plant leaves (Kosma et al., 2014). I hypothesized that the combination of suberin and cutin in the leaves will reinforce defense against pathogens and predicted that overexpression of suberin in leaves would result in reduced susceptibility to attack by *Pst hrcC*$^{-}$ and *B. cinerea*. I also hypothesised that overexpression of suberin in leaves would also result in reduction in overall leaf permeability.

4.5.1 Summary and findings on ectopic expression of suberin in leaves

My results showed that, contrary to my hypotheses, ectopic expression of suberin neither inhibited colonization by bacterial or fungal pathogens nor water loss from the leaves. Greater or equivalent susceptibility of leaves to *Pst hrcC*$^{-}$ and *B. cinerea* was observed in mutant plants ectopically expressing suberin. This finding is evident by the enhanced proliferation of *Pst hrcC*$^{-}$ in the leaves of *AtMYB41 OE-7*, *AtMYB41 OE-10* and *AtMYB41 OE-63* which have elevated leaf suberin, compared to the wild type. Also, the rapid increase in lesions associated with *B. cinerea* infection in overexpression mutant leaves is remarkable.

It is established that the cuticle coating on the plant surface of epidermal cells provides a physical barrier and protective covering against pathogen invasion via the leaves (Cominelli et al., 2008). Suberin is structurally and chemically similar to the cutin contained in plant cuticles. However, cutin is accumulated on the outer surface of the epidermal cell wall of aerial plant organs, whereas suberin is deposited on the inner face of the cell wall adjacent to the plasma membrane (R. B. Franke et al., 2012; Vishwanath et al., 2015). Previously, increased susceptibility to infection by *B. cinerea* was observed in assays using fruits of *cutin deficient* mutants of tomato (Isaacson et al., 2009). Conversely, fungal pathogens have been shown to be able to breach the cuticle using a combination of enzymatic degradation and mechanical rupture (Yeats & Rose, 2013). Though I expected resistance responses to *B. cinerea* by the overexpressed suberin mutants, it is striking to notice that the leaves of both *B. cinerea* infected and uninfected control plants displayed chlorotic and dryness symptoms. It is possible that ectopic expression of suberin results in water loss and plays a significant role in the disease symptoms observed. The further

characterization of leaf permeability of the overexpression mutants clarified that leaves of overexpression mutants with increased suberin deposition (*AtMYB41 OE-7, AtMYB41 OE-10* and *AtMYB41 OE-63)* showed greater permeability than leaves of the wild-type plant. It is possible that ectopic expression of suberin in the Arabidopsis leaves disturbed the integrity of the leaf cuticle and increased permeability, which may, in turn, result in increased nutrient availability to the pathogen.

4.6 Future directions

Suberin deposition in Arabidopsis root may or may not have a role in defense against attack by *F. avenaceum.* To date, our understanding of pathogen defense in the root system is less well understood than in leaf systems, where most research has placed attention. Consequently, there are many questions regarding mechanisms at the cellular and molecular levels of pathogen defense in plant roots. Understanding immunity in the root system is of critical importance in developing newer methods for controlling root diseases and improving crop quality generally by disease prevention in plants. In the future, additional knowledge on this topic may guide crop improvement by genetic engineering. There is a need for additional work at the transcriptomic and proteomic levels of defense responses in Arabidopsis roots upon *F. avenaceum* infection. In particular, it is necessary to disentangle the effects of suberin as a barrier to pathogen invasion and as a barrier to water loss.

Additional insights may be gained from use of overexpression of Arabidopsis *CYP86A1* in roots to study response to *F.* avenaceum and check for improved resistance to this model root pathogen. Efforts should be made to develop additional model pathogen systems for Arabidopsis. My experience indicated that *Pseudomonas* provides an excellent

model for leaf pathogen, but more work needs to be done to develop root pathogen systems. My investigation on the *35S* promoter-driven overexpression of *AtMYB41* that leads to ectopic accumulation of suberin in leaves of Arabidopsis suggests an antagonistic relationship may exist between suberin accumulation in Arabidopsis leaves with and cutin deposition. It will be interesting to study the effect of mutants of Arabidopsis deficient in cutin, but with overexpressed suberin accumulation. My work focuses on sterile systems, and since plants live in natural environment, similar work needs to be done in natural systems**.**

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