Investigating a role for *BLADE-ON-PETIOLE* genes in plant defense

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ABSTRACT

Plants have evolved multiple layers of defense to ward off pathogens. Pathogen-associated molecular pattern (PAMPs)-triggered immunity (PTI) is the most ancient and basal form of plant defense. BLADE-ON-PETIOLE1/2 (BOP1/2) are members of an evolutionarily conserved subclade of BTB-ankyrin proteins that control plant development within the NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) family of plant defense regulators that control systemic acquired resistance. I analyzed the transcriptome of *BOP1* overexpressing plants to find a previously undiscovered role for BOPs in plant defense. Gene Ontology analysis revealed that 35% of upregulated genes belong to plant defense pathways with PTI forming the largest group. Among these PTI genes were clade I TGA factors, also essential for BOP-mediated developmental processes. Data validation and direct pathogen testing support the model that BOPs and clade I TGAs co-regulate a subset of genes involved in PTI. These data uncover a dual role for Arabidopsis BOPs in development and defense.

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A big shout out to my family and friends who have helped and guided me every step of the way. I love you all and hope you understand/appreciate all of my hard work!

PREFACE

This thesis explores the role of organ boundary genes in plant defense, using the plant model species *Arabidopsis thaliana.*

I carried out the majority of work described in this thesis, under the supervision of Dr. Shelley Hepworth who conceived and designed this project and edited my thesis. I would also like to thank Ying Wang for his preliminary analysis of defense gene expression that served as the basis for my project and Bodunde Oyetoran for pathogen assay experiments that validate the biological relevance of my expression data findings.

None of work described in this thesis has been submitted for publication.

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Gain-of-function mutant (dominant): *bop1-6D*

Double mutant: *bop1 bop2*

LIST OF ABBREVIATIONS

AZ – abscission zone

BIK- BOTRYTIS-INDUCED KINASE

BOP - BLADE-ON-PETIOLE

bZIP – basic leucine zipper

CBP- CALMODULIN-BINDING PROTEIN

CDPK – CALCIUM DEPENDENT PROTEIN KINASE

Col – Columbia

DAMP – Damage associated molecular pattern

ET - ethylene

EVR - EVERSHED

FLS – FLAGELLIN-SENSITIVE

FRK1 – FLG22-INDUCED RECEPTOR-LIKE KINASE

GA – gibberellin

GO – Gene Ontology

GLS- GLUCAN SYNTHASE-LIKE

GUS – β-Glucuronidase

JA – jasmonic acid

IDA – INFLORESCENCE DEFICIENT IN ABSCISSION

MAMP – microbe associated molecular pattern

MAPK – MITOGEN ACTIVATED PROTEIN KINASE

MKK – MITOGEN-ACTIVATED PROTEIN KINASE KINASE

NEVER- NEVERSHED

NHL10 – NDR1/HIN1-LIKE 10

NPR1 – NON-EXPRESSER OF PATHOGENESIS RELATED GENE

PAMP – pathogen associated molecular pattern

PTI – PAMPs triggered immunity

PR – PATHOGENESIS-RELATED

RBOHD – RESPIRATORY BURST OXIDASE HOMOLOGUE D

ROS – reactive oxygen species

SA – salicylic acid

SAR – systemic acquired resistance

SARD- SAR DEFICIENT

SDS – sodium dodecyl sulphate

TGA – TGACG

UDP – uridine diphosphate

WT – wild-type

WRKY – WRKY domain-containing

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CHAPTER 1: INTRODUCTION

1.1 Thesis overview

Arabidopsis thaliana (Arabidopsis) is a member of the Brassicaceae family, which includes important agricultural crops such as mustard and canola. Arabidopsis, like other economically important crops, is targeted by a large spectrum of pathogens including fungi, insects, and bacteria. Crop loss associated with pest damage threatens the income of rural families, agricultural companies, and more importantly, threatens food security world-wide (Cerda *et al.*, 2017). Researchers and breeders seek to understand the mechanisms behind plant defense against pathogens in order to generate crops that are resistant to pest damage and high yielding.

Plants have a sophisticated immune system involving multiple layers of defense. In brief, disease resistance in plants starts with the recognition of exterior pathogens leading to defense mechanisms that best limit the infection (Schenk *et al.*, 2000). There exist both localized defense responses and systemic defense responses that plants use depending on the pathogen. In Arabidopsis, the BTB-ankyrin protein NONEXPRESSER OF PATHOGENESIS RELATED GENES1 (NPR1) is a master regulator of systemic acquired resistance (SAR), a type of broad-spectrum immunity initiated plant-wide after a local infection (Cao *et al.*, 1997; Spoel & Dong, 2012). Other members of this family, BLADE-ON-PETIOLE1 and 2 (BOP1/2), function in plant development through interactions with the same family of transcription factors as NPR1 (Khan *et al.*, 2014). My thesis investigates a function for BOP1/2 in plant defense.

1.2 *Arabidopsis thaliana* as a model species for plant biology studies

Mouse ear cress (Arabidopsis) has become the model organism of choice for plant biology. Arabidopsis is a small dicotyledonous plant in the mustard family, closely related to vegetable crops like cabbage and oilseed crops like canola (Meinke *et al.*, 1998). The use of Arabidopsis as

a genetic model for plant research dates back to the early 1980's (Provart *et al.*, 2016). Arabidopsis was selected for research because of features that make it easy to grow in a lab. The plants are small and have a short generation time of approximately 6 weeks from germination to seed dispersal. The genome of Arabidopsis is approximately 20 000 genes organized into five chromosomes. This small genome along with prolific seed production by self- or cross-fertilization is ideal for genetic studies (Meinke *et al.*, 1998; Provart *et al.*, 2016).

Arabidopsis research continues to dominate over crop plants with more obvious economical and agricultural advantages because of an underlying need to understand the fundamentals of plant biology. Inducing gene mutations in Arabidopsis is simple using chemical mutagens, *Agrobacterium*-mediated approaches, and modern genome editing technologies like CRISPR-Cas9 (Bedell *et al.*, 2012; Feng *et al.*, 2014; Provart *et al.*, 2016). Furthermore, the Arabidopsis genome is completely annotated (Arabidopsis Genome initiative, 2000) and stored in one of the largest publicly available databases in the world. This database contains sequence data, characterized mutations, and transgenic lines where the gene mutations interfering with aspects of plant growth and development, metabolic and signal transduction, responses to environmental and hormonal signals, pathogen and other abiotic and biotic stress signals, have all been identified (Somerville & Koornneef, 2002). In terms of plant defense, Arabidopsis was the first species used to isolate plant immune receptor genes, elucidate hormone pathways, and develop the concept of basal defense. The use of Arabidopsis as a model organism has accelerated research on other plant systems and has allowed us to identify and improve key crop traits including resistance to abiotic stresses such as drought and salinity, biotic stresses such as pathogens and pest resistance, and photosynthetic and physiological efficiencies resulting in overall improvements in crop production and yield (Ferrier *et al.*, 2011; Provart *et al.*, 2016).

1.3 Overview of the plant immune system

Plants are continuously exposed to a wide array of different pathogens. To properly detect pathogens and defend against invasion and colonization, plants employ both innate and induced immune systems (Jones & Dangl, 2006). When discussing plant defense, no one general mechanism surfaces, rather plants have evolved multiple different layers of defense. Plant defense begins with predefined, natural barriers that deter pathogen invasion, such as the plant cell wall and extracellular surface coatings like the cuticle (Grennan, 2006). When a pathogen overcomes these physical barriers, plants employ both localized and systemic immune responses to detect and implement a response that prevents further colonization and growth, often tailored to the nature of the invading pathogen (Nümberger *et al.*, 2004; Grennan, 2006)

1.4 Passive outer barriers

The cuticle is a waxy coating on the exterior surface of plant epidermal cells that serves as barrier to protect against water loss and various abiotic and biotic stresses (Fig. 1.1). The cuticle is composed of a polyester called cutin that is a mixture of very long-chain fatty acids and their derivatives interspersed with waxes (Serrano *et al.*, 2014). This substance is embedded with antimicrobials and also the site of production for fungitoxic substances (Grennan, 2006; Chassot *et al.*, 2008; Serrano *et al.*, 2014).

The cell wall is an additional external barrier that plants have evolved and it functions primarily to prevent the entry of invaders who were successful in bypassing the plant exterior surfaces (Fig. 1.1). The primary cell wall consists of carbohydrate-based polymers (cellulose, hemicellulose and pectins) as well as flexible rod-like extensins and other proteins (Grennan, 2006). Throughout development, some cells have the need to reinforce their structure and go through cellular expansion in which a secondary cell wall is generated composed of cellulose and

hemicelluloses like xylans as well as lignin (Cosgrove, 2005; Sarkar *et al.*, 2009; Miedes *et al.*, 2014). The structure of the cell wall allows it to function as a passive barrier because pathogens are required to break down the cell wall matrix for successful infection and colonization. When the cell wall is degraded, the plant dips into the reservoir of antimicrobial compounds located in the cell wall aiding in the prevention of pathogen invasion. Most importantly, plants have a special mechanism known as the cell wall integrity maintenance mechanism (similar to fungi) that is triggered by external signals such as pathogen attack or wounding. Special signalling molecules known as Damage-Associated Molecular Patterns (DAMPs) are produced and modulate plant immune responses in a similar mechanism to PAMPS-triggered immunity (PTI) (Vorwerk *et al.*, 2004; Cantu *et al.*, 2008; Miedes *et al.*, 2014).

1.5 Localized responses

Pathogens capable of penetrating the preformed outer barriers still have to face the plant immune system. To prevent pathogen invasion, plants are capable of producing localized immune responses at the site of recognition (Fig. 1.2a). This innate immunity system relies on localized surface immune receptors that recognize conserved regions of microbes known as pattern associated molecular patterns (PAMPs) and this leads to a local defense response known as PTI (Spoel & Dong, 2012; Henry *et al.*, 2013). These receptors are also capable of recognizing DAMPs and initiate a similar mechanism. In the evolutionary battle between plants and microbes, pathogens have evolved a set of proteins and other specialized macromolecules called effectors that overcome this PTI response. To detect specialized pathogens that are capable of surpassing these surface receptors, resistant plants employ intracellular immune receptors that recognize effector proteins from the pathogens and this leads to a robust local response known as effector-triggered immunity (ETI, Fig. 1.2b). These localized immune responses function not only to

prevent pathogen invasion, but they also function as a priming mechanism, enabling the plant to have heightened resistance against future attacks (Henry *et al.*, 2013). ETI also functions as mechanism to warn distal parts of the plant against future attacks through the production of mobile signals that lead to a systemic response (Fig. 1.2c).

1.6 Systemic responses

When a pathogen is present, plants establish a network of cross-talk between responses that allow the plant to differentiate and determine whether the invading pathogen is biotrophic (feeds on living host tissue) or necrotrophic (kills host tissue and feeds on the remains) so the most effective defense mechanism is amplified (Glazebrook, 2005). This cross-talk between defense responses depends on the levels of major signalling molecules and functions to alert distal tissue of the pathogen detection. When biotrophic pathogens are recognized, salicylic acid (SA) dependent signalling is the dominant response resulting in the activation of signature defense genes like *PATHOGENESIS-RELATED GENE1 (PR1)*. The activation of SA-mediated pathways results in a long-lasting systemic defense known as SAR (systemic acquired resistance) whose key regulator is NPR1 (Cao *et al.*, 1997; Dong, 2004; Conrath, 2006; Bigeard *et al.*, 2015). In contrast, when a chewing insect or necrotrophic pathogens are recognized, the production of jasmonic acid (JA) and ethylene (ET) are required for signalling to induce a very different type of response (Glazebrook, 2005; Vlot *et al.*, 2009; Pieterse *et al.*, 2012). The interaction between these opposing pathways have been extensively studied, suggesting many nodes of cross talk are present for proper regulation. The interaction between SA and JA hormone pathways can be synergistic, neutral, or antagonistic with the latter obtaining the most support. (Glazebrook, 2005; Pieterse *et al.*, 2012).

1.7 Innate immunity as the first line of defense

The first and most ancient line of plant defense regulated by the immune system is innate immunity, often referred to as basal resistance. This form of immunity is not novel to plant systems, it is found in fungi and animal systems as well (Uehling *et al.*, 2017). This form of immunity is evolutionarily important and its hallmarks and components can be traced back to liverworts and mosses such as *Physcomitrella patens*. These are among the first plants that colonized land and have acquired defense mechanism such as basal immunity to fight pre-existing microorganisms (Ponce de León & Montesano, 2017).

1.7.1 Pathogen associated molecular patterns (PAMPs)

Innate immunity in plants is also called PAMPs-triggered immunity (PTI) since this immune response relies on the exterior recognition of PAMPs or in cases where the microbe is not disease-causing, microbial-associated molecular patterns (MAMPs) (Bigeard *et al.*, 2015). PAMPs are conserved protein regions of pathogen microbes such as bacterial flagellin, elongation factors, peptidoglycan, and lipopolysaccharides (Nümberger *et al.*, 2004; Grennan, 2006). PAMPs are recognized by specific pattern recognition receptors within the plasma membrane of plant cells leading to a complex response as depicted in Fig. 1.3.

1.7.2 Receptors

To date, a few different pattern recognition receptors and corresponding PAMPs have been identified, the most characterized being the Arabidopsis FLAGELLIN SENSITIVE2 (FLS2) receptor that is responsible for recognizing the N-terminus of flagellin from the bacteria *Pseudomonas aeruginosa*, specifically a 22-amino-acid-long epitope called flg22 (Gómez-Gómez & Boller, 2000; Bigeard *et al.*, 2015). In the FLS2 receptor pathway, the receptor-like cytoplasmic kinase, BOTRYTIS-INDUCED KINASE1 (BIK1), is rapidly phosphorylated and released from

the receptor complex upon pathogen perception (Bigeard *et al.*, 2015). Other common PAMPs are elongation factor thermo unstable (Tu) and fungal chitin, which are recognized by ELONGATION FACTOR TU RECEPTOR1 (EFR1) and LYSINE MOTIF-CONTAINING RECEPTOR-LIKE KINASE1/CHITIN ELICITOR RECEPTOR KINASE1 (LYK1/CERK1) respectively (Gómez-Gómez & Boller, 2000; Zipfel *et al.*, 2006).

1.7.3 Early responses

The earliest known physiological response after pathogen perception is changed ion flux across the plasma membrane. This ion flux results in a rapid and transient alkalinisation of the plasma membrane leading to an increase in cytosolic Ca^{2+} which acts as a second messenger and is positively regulated by proteins such as BIK1, which lead to a signal transduction mechanism (Zipfel & Robatzek, 2010). In Arabidopsis, around 2-3 minutes after pathogen perception, an early hallmark of the PTI response initiated: a burst of reactive oxygen species (ROS) which peaks anywhere between 6-10 minutes. This rapid and transient ROS burst is created in part by a plasma membrane NADPH oxidase induced by PAMPs known as respiratory burst oxidase homolog D (RBOHD) (Nühse *et al.*, 2007; Ranf *et al.*, 2011). This RBOHD produces a superoxide anion (O_2) in the apoplast (extracellular space) which is membrane impermeable and converted into hydrogen peroxide (H_2O_2) by superoxide dismutase, now capable of entering the cytosol where it can play a signaling role (Ranf *et al.*, 2011).

The next aspect of the PTI response involves multiple families of proteins included in signal transduction. Kinases and phosphatases play a central role in rapid/reversible post translational modifications important for signal transduction. Among these, calcium-dependent protein kinases (CDPKs) aid in transcriptional reprogramming and mitogen-activated protein kinases (MAPKs) help translate extracellular stimuli into appropriate intercellular responses

(Bigeard *et al.*, 2015). In Arabidopsis, there are 20 MAPKs, 10 MAPKKs, and 60 MAPKKKs (Ichimura *et al.*, 2002; Bigeard *et al.*, 2015). Of these, at least four MAPKs (3,4,6 and 11) are important for initiating various PTI signalling modules however the molecular details remains cloudy (Bigeard *et al.*, 2015). Almost half of all MAPK kinase substrates are transcription factors, including bZIP factors and several WRKY proteins activated by phosphorylation (Bigeard *et al.*, 2015).

Another aspect of the PTI pathway that is initiated within the first hour of pathogen perception is the closure of stomata. The stomata consist of a pair of guard cells (specialized epidermal cells) that regulate gas exchange and water loss. Stomata serve as a natural opening to invading pathogens and 1 hour after perception, stomata begin to close (Melotto *et al.*, 2008).

1.7.4 Late responses

Immune signaling ultimately leads to the activation of defense-related genes that reinforce the outer barriers to prevent pathogen invasion and colonization. There are various classes of defense genes that are activated that function in similar pathways in order to limit pathogen invasion and growth. Some of these families include genes involved in antimicrobial compound synthesis, marker defense genes such as *PR1,* or defense genes specific to the PTI pathway such as *FLG22-INDUCED RECEPTOR-LIKE KINASE1* (*FRK1*) and *ARABIDOPSIS NON-RACE SPECIFIC DISEASE RESISTANCE/TOBACCO HAIRPIN-INDUCED GENE-LIKE10* (*NHL10*) (Bigeard et al., 2015). Other studies have identified genes associated with photosynthesis-related processes to be significantly downregulated in response to PAMP perception. By repressing the presence of these photosynthetic genes, the plant is ultimately cutting off or limiting the resources that are available for pathogens to capitalize on. This serves as a mechanism to prevent further microbial growth or colonization (Li *et al.*, 2016). This also highlights a link to a common late

response seen as part of the PTI pathway, which is growth inhibition, where pathogen perception significantly reduces the growth of plants (Huot *et al.*, 2014).

Another late hallmark of the PTI response is the deposition of a callose at sites of injury in the plant cell wall. Callose is a polymer of glucose joined by β-1,3 linkages. GLUCAN SYNTHASE5 (GSL5) is a callose synthase in Arabidopsis (Jacobs *et al.*, 2003; Ellinger & Voigt, 2014). Callose synthases form callose using UDP-glucose as a substrate beginning around 12 hours after pathogen perception in Arabidopsis (Li *et al.*, 2016). Various callose biosynthetic genes are induced by PAMP perception, as well by other plant growth and environmental cues such as the production of ROS by RBOHD (Wu *et al.*, 2014).

One of the most effective ways to study plant defense is to use tools that allow researchers to query the whole genome of a plant rather than focusing on one individual gene at a time (Moustafa & Cross, 2016). The cDNA microarray gives insight into gene expression profiles of thousands of genes simultaneously, allowing researchers to study gene levels in different defense responses (Eisen *et al.*, 1998; Schenk *et al.*, 2000). In Arabidopsis, past studies have focused on gene expression profiling during PTI, JA/ET-mediated, and SA-mediated defense responses (AbuQamar *et al.*, 2006; Wan *et al.*, 2008; Blanco *et al.*, 2009). The transcriptome profiles of such responses are useful for assigning putative roles to genes as well as for identifying gene functions under different conditions (Schenk *et al.*, 2000). Large scale transcriptome studies of the various defense responses also provide large datasets of differentially regulated genes which then open opportunities for others to build on this data to find novel players in plant defense. The use of bioinformatics tools such as Gene Ontology (GO, http://www.geneontology.org) enrichment analysis are extremely effective in elucidating the functional role that the various differentially-regulated genes have in specific responses (Ashburner *et al.*, 2000).

1.8 Role of BTB-ankyrin proteins in defense and development

BTB-ankyrin proteins are a family of plant-specific co-transcription factors first discovered in Arabidopsis that regulate both development and defense (Khan *et al.*, 2014). Proteins in this family function as adaptor-like transcriptional co-activators characterized by an N-terminal BTB/POZ (for Broad-Complex, Tramtrack, and Bric-a-brac/POX virus and zinc finger) domain and a series of four ankyrin repeats towards the C-terminus (Khan *et al.*, 2014). Both motifs are used for protein-protein interaction. The BTB/POZ domain mediates dimerization and interacts with CULLIN3 to target proteins for degradation (Jun *et al.*, 2010; Fu *et al.*, 2012; Zhang *et al.*, 2017; Chahtane *et al.*, 2018). The ankyrin repeats interact with TGA (TGACG-motif binding) basic leucine zipper (bZIP) transcription factors for recruitment to DNA (Zhang *et al.*, 1999; Després *et al.*, 2000; Zhou *et al.*, 2000; Hepworth *et al.*, 2005). In Arabidopsis, this family contains a primary subclade comprised of NPR1 and three close homologs (NPR2, NPR3, and NPR4) with roles in SAR (Cao *et al.*, 1997; Fu *et al.*, 2012) and a secondary subclade comprised of BOP1 and BOP2 with roles in plant development as outlined in Fig 1.4 (Khan *et al.*, 2014).

1.8.1 NPR clade regulates plant defense

A role for NPR1 in plant defense was first identified based on loss-of-function mutations that eliminated SAR (Cao *et al.*, 1994; Cao *et al.*, 1997). NPR3 and NPR4 also play a role in this response (Fu *et al.*, 2012; Yan & Dong, 2014; Ding *et al.*, 2018). SAR is a long lasting, broad spectrum defense response mediated by SA (Vlot *et al.*, 2009). Following local infection, elevated levels of SA lead to enzymatic reduction of NPR1 to a monomeric state, allowing its translocation into the nucleus. Inside the nucleus, NPR1 interacts with TGA bZIP transcription factors, allowing the induction of gene expression to induce SAR (Mukhtar *et al.*, 2009). NPR1 interactions with clade II TGAs (TGA2,TGA5 and TGA6) play a major role since *tga2 tga5 tga6* triple mutants lack

a SAR response similar to *npr1* mutants (Cao *et al.*, 1994; Zhang *et al.*, 2003). NPR1 also interacts with clade III TGA3 (Choi *et al.*, 2010) and with reduced forms of clade I TGA1 and TGA4 to impart its function (Després *et al.*, 2003; Sun *et al.*, 2017).

1.8.2 BOP clade regulates plant development

Unlike NPR1, BOP1/2 regulate plant development. BOP1/2 expression is enriched at meristem-organ boundaries. Boundaries are zones of restricted growth that control organ separation and the morphogenesis of leaves and flowers. Boundaries are also sites for the production of axillary meristems and formation of abscission zones. Patterning of all of these structures is impaired in *bop1 bop2* mutants (Khan *et al.*, 2014; Hepworth & Pautot, 2015). *BOP1/2* are also expressed in primary and secondary vasculature tissues of roots and promote symbiotic nodule organ identity in legumes (Couzigou *et al.*, 2012; Liebsch *et al.*, 2014; Woerlen *et al.*, 2017). BOP1/2 interactions with clade V TGA8/PAN are important for floral patterning (Hepworth *et al.*, 2005; Xu *et al.*, 2010) but clade I TGA1 and TGA4 play a more central role (Wang *et al.*, 2018). *TGA1* and *TGA4* are expressed at organ boundaries and function in the same genetic pathways as BOP1/2 required for SAM maintenance, flowering, and inflorescence architecture. These are interesting findings given that TGA1 and TGA4 have a more characterized role in innate plant immunity. Loss-of-function *tga1 tga4* mutants are deficient in basal resistance to bacterial pathogens, exhibiting reduced apoplastic defenses and impaired SA biosynthesis (Kesarwani *et al.*, 2007; Shearer *et al.*, 2012; Wang & Fobert, 2013). Collectively, these data raise several questions. Do *BOP* genes also play a dual role in plant development and plant defense? And if so, do they function in the same genetic pathway as the clade I TGAs for both roles?

1.9 Defense and development

The activation of defense mechanisms in plants is an energetically costly process that occurs at the expense of growth (Huot *et al.*, 2014). This area of 'growth-defense trade-off' is a highly active field of research. Plants actively expressing defense responses grow more slowly and reproduction can be repressed (Coley *et al.*, 1985; Huot *et al.*, 2014). For centuries, crops have been bred to maximize growth-related traits and this may have led to the emergence of genotypes that compromise the balance of defense and growth, possibly leading to more disease susceptible crops (Strange & Scott, 2005). A prominent physiological consequence resulting from prolonged expression of the PTI defense pathway is growth inhibition. Treating a plant with a PAMP for a long duration results in a strong growth inhibition (Gomez-Gomez *et a*l., 1999, Huot *et al*., 2014 Zipfel *et al.,* 2006). Although this differs from a natural environment where the exposure to PTI elicitors is limited and often not prolonged or continuous, these studies allow us to benefit from the developmental defects associated with prolonged defense responses. Understanding the mechanisms that plants use to balance growth and defense is therefore extremely important from the perspective of agriculture. We hypothesize that *BOP* genes play a role in this regulation given their proximity to *NPR1* in the evolution of land plants.

Auto-immune mutants are a class of mutants that are permanently expressing defense responses (Fig. 1.5). These mutants tend to exhibit a dwarf phenotype because they constitutively express defense genes and show broad-spectrum resistance to pathogens. Other characteristics of auto-immune mutants are include the elevation of defense hormones (SA, JA), accumulation of callose deposits, and ROS production (van Wersch *et al.*, 2016). Plants overexpressing *BOP1* display at least some of these features: they are dwarf and late flowering with elevated levels of

JA (Norberg et al. 2005; Khan et al. 2012b; Khan et al. 2014). Further exploring these links may lead to useful insights in understanding development-defense trade-offs and how they work.

1.10 Defense and abscission

Abscission is a natural process in plants that allows organs to detach in response to development or stress signals (Patharkar & Walker, 2017). Abscission zones are typically located at boundaries where organs are joined to the plant body. This process can be broken down into four stages. 1) formation of an abscission zone, 2) abscission zone cells gain the ability to respond to abscission-promoting signals, 3) activation of abscission resulting in organ separation, and 4) formation of a protective layer over exposed cells on the plant body to prevent water loss and pathogen invasion as seen in Fig. 1.6 (Estornell *et al.*, 2013).

Interestingly, abscission and innate immunity signaling pathways involve the use of similar hormone signals, membrane receptors, signal transduction machinery, and downstream activation of enzymes that modify and reinforce the cell wall (Meir *et al.*, 2011; Patharkar & Walker, 2017). In particular, the last step of abscission is hypothesized to be a defense mechanism (del Campillo & Lewis, 1992; Lee *et al.*, 2018). Several studies have noted the upregulation of defense genes such as the *PR* gene family in abscission zones during abscission (del Campillo & Lewis, 1992; Meir *et al.*, 2011; González-Carranza *et al.*, 2012; Kim *et al.*, 2015). Many plants including Arabidopsis use abscission to discard organs that become damaged by insect feeding or disease (Patharkar & Walker, 2015; Patharkar & Walker, 2017). This mechanism promotes survival by giving a plant sufficient time and resources to mount an effective immune response (Faeth *et al.*, 1981; Patharkar & Walker, 2017). In Arabidopsis, key players involved in abscission signaling including INFLORESCENCE DEFICIENT IN ABSCISSION, HAESA, and NEVERSHED are also required during pathogen-triggered abscission. However, treatment with a pathogen to trigger

PTI defense did not result in constitutive abscission suggesting the need for the delivery of effector proteins within the cells to trigger this mechanism (Patharkar & Walker, 2016).

BOP1/2 activity appears to be required throughout the abscission process, beginning with abscission zone differentiation. Mutations in *bop1 bop2* block formation of specialized cells required for abscission so that organs are never shed (McKim *et al.*, 2008). Conversely, abscission is accelerated in transgenic plants that overexpress *BOP1* (Corrigan, 2018). BOP1/2 were recently shown to regulate the production of ROS in the AZ needed for signaling and polymerization of lignin required for separation. Indeed, *rbohD rbohF* double mutants plants with depleted ROS are unable to abscise the floral organs resulting in a defect similar to *bop1 bop2* mutants (Lee *et al.*, 2018). This new study sheds light on the link between abscission, defense, and how BOPs might function in both.

1.11 Research question: a role for boundary genes in plant defense?

In summary, several major pieces of evidence suggest that BOPs have an alternative role in plant defense. First, BOPs are closely related to NPR1 and interact with several of the same bZIP transcription factors to exert their function (Khan *et al.*, 2014; Wang *et al.*, 2018). Among these, clade I TGA bZIP transcription factors contributing both to SA biosynthesis and innate immunity are essential for BOP-dependent regulation of plant development (Sun *et al.*, 2017; Wang *et al.*, 2018). Second, plants overexpressing BOPs are dwarfed and accumulate high levels of jasmonic acid (Khan *et al.*, 2015) similar to autoimmune mutants (van Wersch *et al.*, 2016). Third, BOPs are essential for abscission (McKim *et al.*, 2008) which is proposed to contain a built-in defense module against pathogen colonization (Estornell *et al.*, 2013; Lee *et al.*, 2018). My thesis directly tests the hypothesis that BOPs play a role in plant defense.

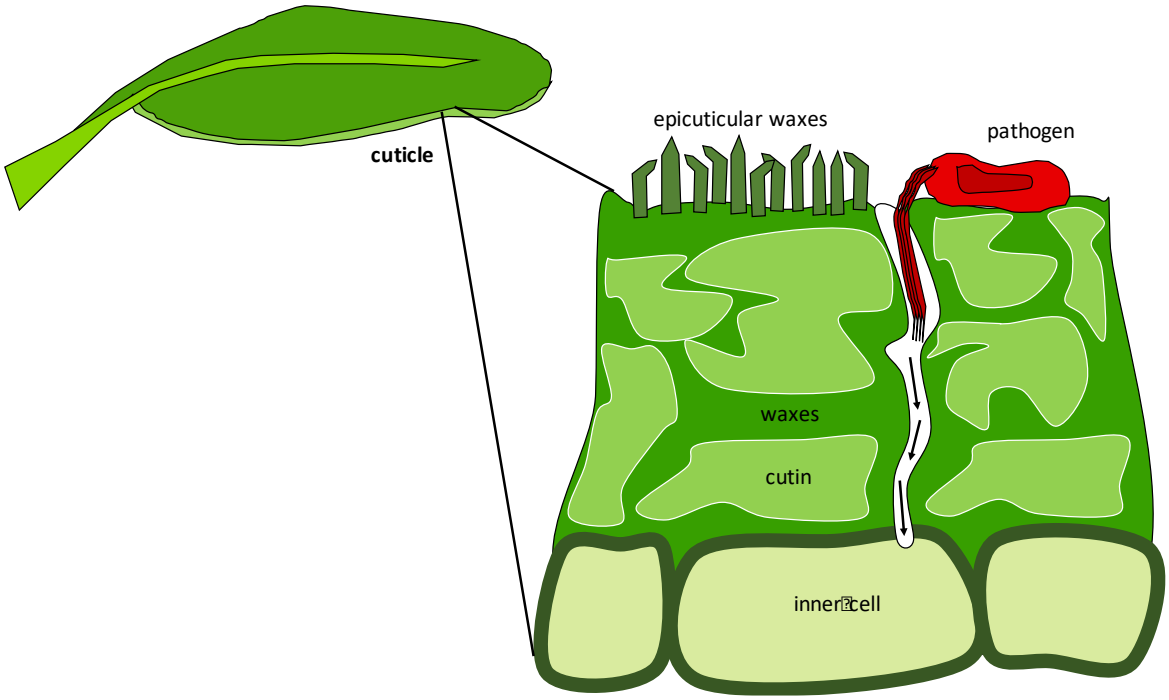

Figure 1.1 Schematic depicting the passive outer barriers plants have evolved.

Some of the morphological traits plants have adapted as a means to prevent pathogen invasion include plant cell walls and the cuticle, a surface layer composed of cutin embedded with waxes. These barriers can be embedded with other defense molecules including antimicrobial compounds.

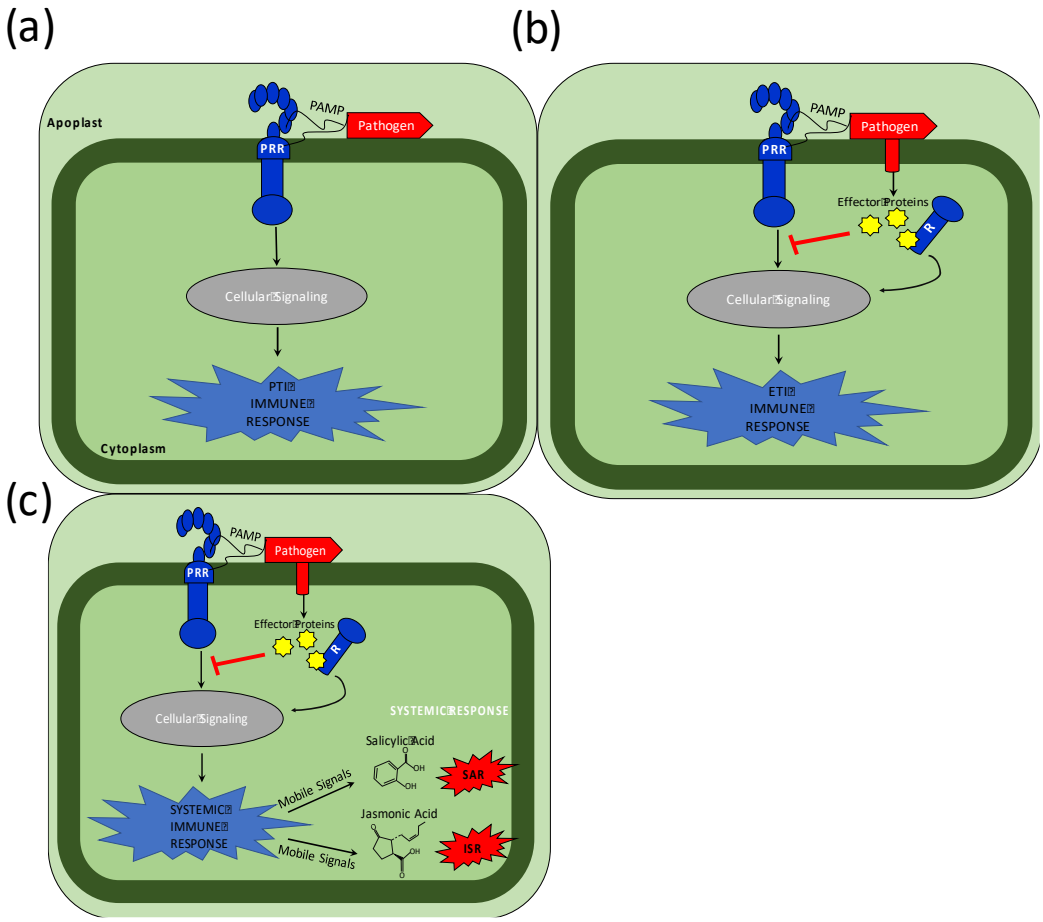

Figure 1.2 Schematic showing the different tiers of plant defense.

(a) The PTI defense response relies on the recognition of a PAMP by specialized plant receptors in the plasma membrane which initiates the PTI pathway. (b) Pathogens are able to secrete effector proteins within the cell that dismantle the PTI pathway but if recognized by plant receptors within the cell, ETI is triggered. (c) Mobile signals and hormone production released following ETI triggers a systemic immune response which is tailored to the pathogens nature and regulated by different hormones.

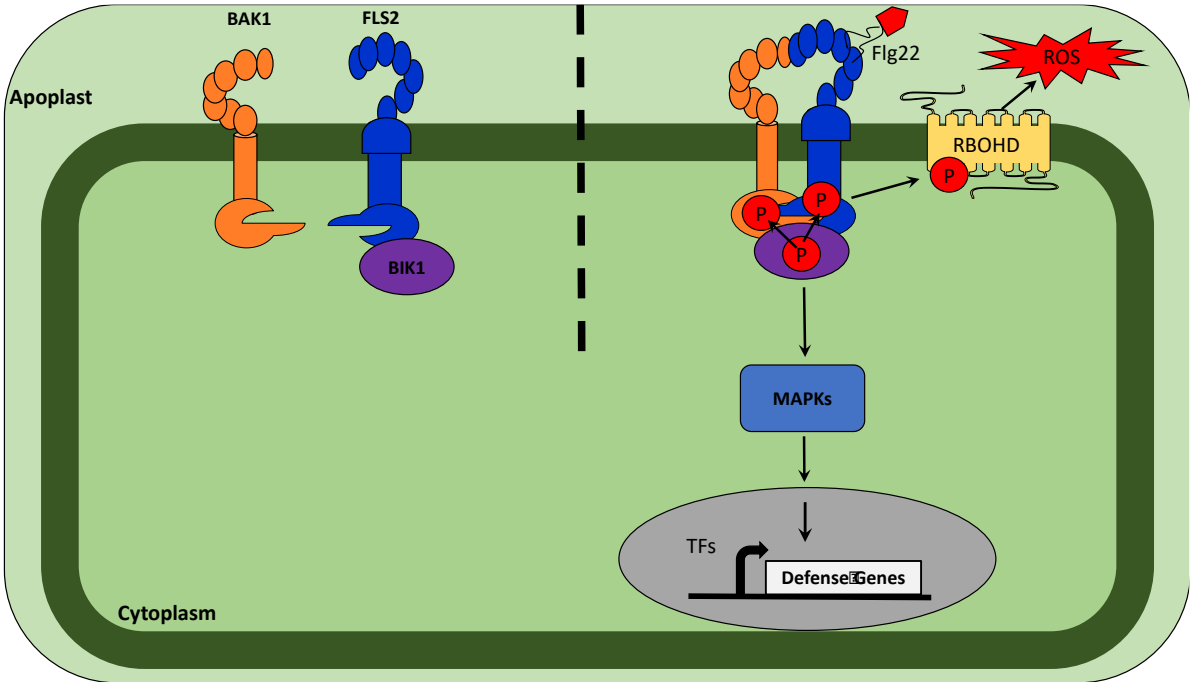

Figure 1.3 Schematic showing a simplified version of the PTI signaling pathway.

(Left) Inactive FLS2 receptor interacts with BIK1. (Right) In the presence of a PAMP (flg22 peptide) FLS2 and its co-receptor BAK1 form a stable heterodimer which leads to the activating phosphorylation of BIK1. BIK1 in turn phosphorylates BAK1 and FLS2 leading to the activation of a MAP kinase (MAPK) signalling cascade resulting in the expression of defense genes. BIK1 phosphorylation of RBOHD leads to a rapid transient burst of reactive oxygen species (ROS) in the apoplast as a second signaling mechanism. P, phosphate group; TF, transcription factor.

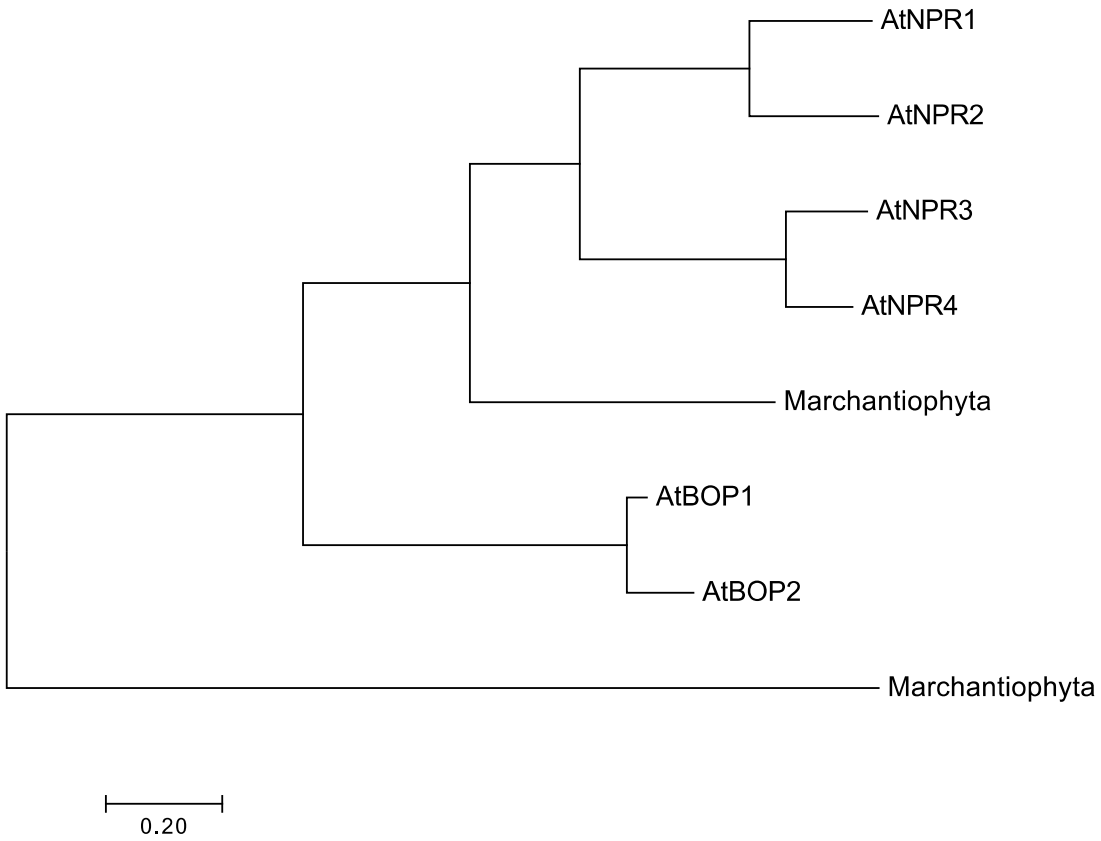

Figure 1.4 Phylogenetic analysis of BTB-ankyrin proteins

Image depicts a phylogenetic analysis inferred by the Maximum likelihood method based on the Poisson correction model. BTB-ankyrin proteins from Arabidopsis (At) and *Marchantia polymorpha* representative of liverworts (Marchantiophyta) were analyzed. The tree is rooted and drawn to scale using MEGA7 (www.megasoftware.net). Branch lengths are proportional to the number of amino acid substitutions per site.

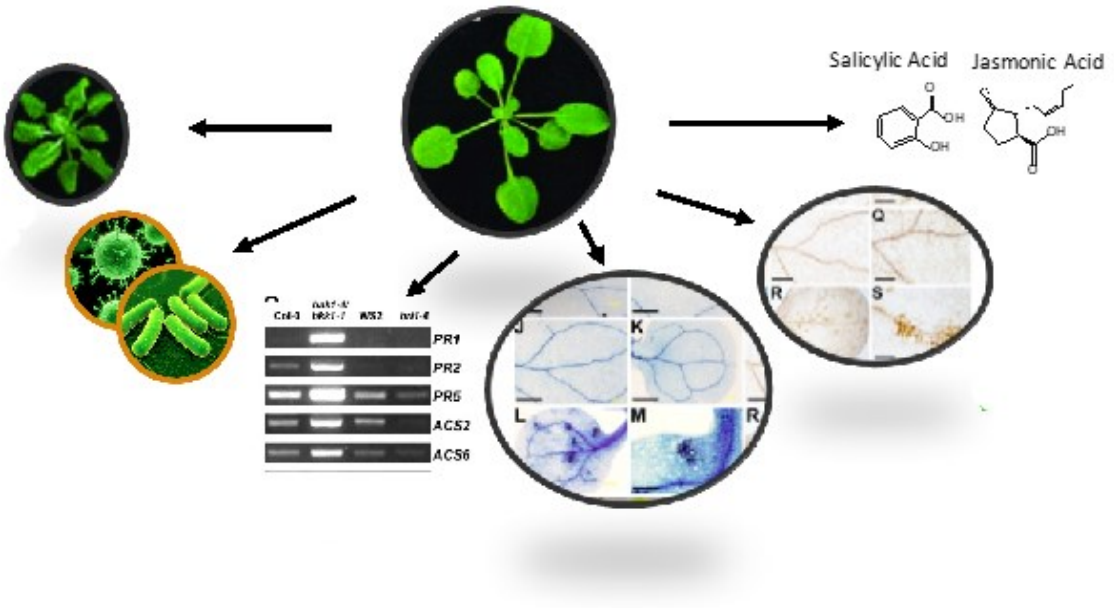

Figure 1.5 Autoimmunity characteristics in plants.

Image depicts the typical phenotypic characteristics associated with autoimmunity in Arabidopsis. From left, autoimmune phenotypes include dwarfism, broad-spectrum resistance to pathogens, heightened defense gene profile under normal conditions, callose deposition and ROS accumulation under normal conditions, and elevated defense signaling hormones such as JA and SA.

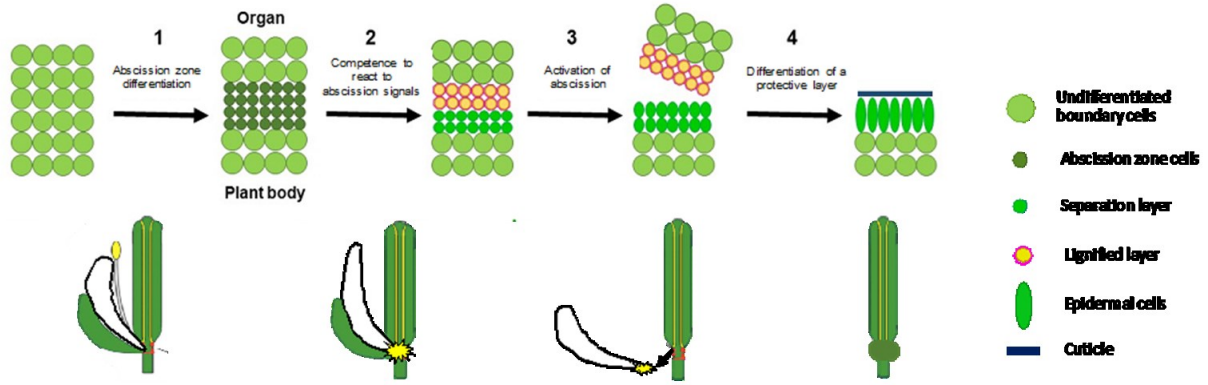

Figure 1.6 Schematic depicting the four stages of abscission.

An overview of the four stages of abscission that occur in order for Arabidopsis to actively shed its floral organs. First, cell undergo differentiation to form the abscission layer, then they receive hormonal or environmental signals that allow for the activation of abscission. The floral organ is then shed revealing an exposed layer that re-differentiates to form epidermal cells and a cuticle to protect from pathogens and water loss. Modified from (Corrigan, 2018).

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant material and growth conditions

The Columbia-0 (Col-0) ecotype of *Arabidopsis thaliana* was used as wild-type. Mutant alleles used in this study were activation-tagged *bop1-6D* overexpression line (Norberg *et al.*, 2005), *bop1-3 bop2-1* double mutant with very low transcript accumulation (Hepworth *et al.*, 2005), *bop1-4 bop2-11* double mutant with no transcript accumulation (Ha *et al.*, 2004), *tga1-1 tga4-1* double mutant (Kesarwani *et al.*, 2007), and *bop1-3 bop2-1 tga1-1 tga4-1* quadruple mutant (Wang *et al.*, 2018). The *fls2-17* mutant used in this study (Zipfel *et al.*, 2004) was kindly provided by Gopal Subramaniam, Agriculture and Agri-Food Canada. The *PR1:GUS* reporter gene line (Shapiro & Zhang, 2001) was kindly provided by Harsh Bais, University of Delaware. Seeds were surface-sterilized prior to sowing as previously described (Popescu, 2018). In brief, seeds were rinsed 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, seeds were rinsed four times in sterile distilled water and sown on agar plates containing minimal media (Haughn & Somerville, 1986) for general propagation of plants or 1% Murashige and Skoog (MS) media for experiments involving seedlings. Plated seeds were wrapped in foil and incubated at 4ºC in the dark for 2-3 days to break dormancy. Following incubation, plates were moved to continuous light for 9-10 days while the seeds germinated. After germination, seedlings were harvested for experimental use or transplanted to 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). Transplants were grown to maturity in growth chambers under continuous light (~115 µmol m^{-2} sec^{-}) at 21ºC.

2.2 Microarray analysis

Microarray analysis was performed as previously described (Khan *et al.*, 2015). In brief, total RNA was harvested from the first expanded internodes of wild-type and *bop1-6D* flowering plants grown under continuous light. RNA samples were prepared for four biological replicates per genotype using an RNeasy Plant Mini Kit (Qiagen Canada, Montreal, QC). Experiments were carried out using Arabidopsis 70-mer oligo microarray slides (http://ag.arizona.edu/microarray). RNA amplification, labelling, hybridization, and scanning were performed as previously described (Xiang *et al.*, 2011; Khan *et al.*, 2015). Data were analyzed using BioConductor tools in R as previously described (Gentleman *et al.*, 2004; Khan *et al.*, 2015).

2.3 Bioinformatics

Differentially expressed genes in *BOP1* overexpressing plants relative to wild-type were sorted into functional categories using Gene Ontology (GO) analysis (Ashburner *et al.*, 2000). A web-based tool called agriGO (bioinfo.cau.edu.cn/agriGO/) was used where upregulated genes were referenced against the Arabidopsis TAIR10 reference genome using default parameters: p-value cutoff of 0.05 as significant using a Fisher statistical test and Yekutieli adjustment for false discovery rate under dependency (Du *et al.*, 2010). Princeton GO (go.princeton.edu) was also used where upregulated genes were referenced against the Arabidopsis TAIR10 reference genome using default parameters: p-value cutoff of 0.01 as significant, Bonferroni correction for multiple comparisons, and adjustment for false discovery rate. These combined tools were used to sort upregulated genes into functional categories and defense pathways.

The relationship between *BOP1* overexpression and plant defense was independently analyzed by directly comparing differentially expressed genes to published microarray data sets representative of PTI, SA-dependent, JA-dependent defense responses. For PTI, a microarray

dataset comparing differentially expressed genes after treatment of Arabidopsis seedlings with three different PAMPs was used (Wan *et al.*, 2008). This dataset was chosen as a representative dataset because Affymetrix ATH1 whole-genome array technology was used to analyze Arabidopsis Col-0 seedlings treated with chitin for 60 minutes and this dataset was overlapped with previously existing microarray datasets of Arabidopsis seedlings exposed to flg22 for 30 minutes and elf26 peptide for 60 minutes to build a consensus dataset (Zipfel *et al.*, 2004; Zipfel *et al.*, 2006; Wan *et al.*, 2008). For SA-mediated plant defense, a microarray dataset showing genes upregulated in response to SA treatment was used (Blanco *et al.*, 2009). This study was chosen as a representative data set since it used microarray technology to analyze gene expression in Arabidopsis Col-0 seedlings after exposure to SA for 2.5 hours and the results were validated by comparing marker gene expression after treatment with a pathogen initiating the same SAR response (Blanco *et al.*, 2009). For JA-mediated plant defense, a microarray dataset showing gene expression changes in response to *Botrytis cinerea* infection was used. This pathogen elicits a strong JA response and was used as a representative data set since it used the same Columbia ecotype and provided a large, complete dataset by RNA-seq which was lacking in other publications for these conditions (Windram *et al.*, 2012). The transcriptome of *BOP1* overexpression plants was also compared to RNA sequencing data from the abscission zone (AZ) of Arabidopsis flowers (Lee *et al.*, 2018). This paper was chosen since previously published datasets were often small, incomplete, and not annotated to updated standards.

2.4 RNA Extraction and qRT-PCR

For expression analysis, total RNA was isolated from 10-day-old seedlings or internodes of flowering plants. Samples were collected for three biological replicates. Each replicate contained pooled tissue from ten or more plants. RNA was prepared using the Plant Total RNA

Mini Kit following the manufacturer's protocol (Geneaid Biotech, New Taipai City, Taiwan). Residual genomic DNA was removed by DNase I digestion of samples according to the manufacturer's instructions (Froggabio Canada, Toronto, ON). The concentration of RNA was quantified using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Markham, ON) using the A260 reading. The quality of the RNA sample was assessed by measuring A260/A280 ratio as well as by running 2 µl of RNA on a 1% agarose gel. Total RNA (1 µg) was reverse transcribed using Invitrogen Superscript III as the polymerase according to the manufacturer's instructions (Thermo Fisher Scientific, Markham, ON). The resulting cDNA was diluted 10-fold and 2 µl was used per each 10 µl reaction volume. qRT-PCR reactions were prepared using Invitrogen Power SYBER Green PCR Master Mix (Thermo Fisher Scientific, Markham, ON) and run using an Applied Biosystems StepOnePlus thermocycler (Thermo Fisher Scientific, Markham, ON). Gene-specific primers were as listed in Table 2.1. Primers were designed manually and spanned an intron-exon junction were possible. Reactions were performed in triplicate. Differences in gene expression, expressed as a fold-change relative to wild-type were calculated as described (Pfaffl, 2001). Values were normalized to *GLYCERALDE-3-PHOSPHATE DEHYDROGENASEC (GAPC)* and *ACTIN1 (ACT1)* for microarray validation experiments (Khan *et al.*, 2015; Sun *et al.*, 2017). Values were normalized to *ACT1* and *UBIQUITIN10 (UBQ10)* for flg22-treatment experiments (see Table 2.1). Data represent mean ± standard deviation (SD) for three biological replicates.

2.5 Seedling growth inhibition assay

Seedling growth inhibition assays were performed on wild-type and homozygous *bop1-6D*, *bop1 bop2*, *tga1 tga4*, *tga1 tga4 bop1 bop2*, and *fls2* mutants essentially as described (Schwessinger *et al.*, 2011; Monaghan *et al.*, 2014). In brief, fresh seeds were surface sterilized

and sown on 1% MS agar plates. Seeds were stratified for 3 days at 4°C and then placed under continuous light at room temperature. On day 4, seedlings were transferred to wells in a sterile flat-bottom 24-well tissue culture plate containing 500 µl of liquid 1% MS media with or without 1 µM flg22 as elicitor (P6622, PhytoTechnology Laboratories, Lenexa, KS). Seedlings were grown for an additional 10 days. After this time, seedlings were removed from wells individually and weighed after gently blotting dry using 10-12 seedlings per replicate. Percent inhibition was calculated by dividing the average fresh weight of mock versus flg22-treated seedlings. Data from 2-4 independent experiments were averaged.

2.6 Quantitative analysis of flg22-induced gene expression

Seedlings were grown on 1% MS agar plates for 6 days. On day 6, seedlings were transferred to wells in a sterile flat bottom 24-well tissue culture plate containing 500 µl of 1% liquid MS. After ten days, seedlings were treated with a solution of 1% MS with or without 1 µM flg22 for 0 hours, 4 hours, and 12 hours (this last time-point was not used). After the designated time, seedlings were removed, blotted dry, and flash-frozen in liquid nitrogen. Three biological replicates were collected. Each replicate contained 10-12 seedlings. Samples were stored at -80 until used for RNA extraction and qPCR as described above.

2.7 Staining for callose deposition

Wild-type and mutant siliques were stained for callose deposition as described (Kim *et al.*, 2005; Clay *et al.*, 2009). Pooled siliques from three plants per genotype were analyzed. Floral organs dissected from the base of *bop1 bop2* siliques to exposed the abscission zone. The siliques were fixed and cleared overnight in a 1:3 solution of acetic acid/ethanol (v/v). The next day, the siliques were washed in 150 mM K_2HPO_4 and then stained with a solution of 0.01% aniline blue in 150 mM K_2HPO_4 in tubes wrapped with aluminum foil for light protection. Stained leaves were

mounted in 50% glycerol and imaged under UV light using a compound microscope (Axio Imager, Carl Zeiss Canada, North York, ON).

2.8 Staining for H_2O_2 accumulation in seedlings

H_2O_2 production in wild-type and mutants was monitored by diaminobenzidine (DAB) staining as previously described (Thordal-Christensen *et al.*, 1997). In brief, seedlings were grown on minimal media plates under continuous light at room temperature until they were 10-days-old. Whole seedlings and dissected rosette leaves were placed in wells of a flat-bottom 24-well tissue culture plate containing 400 µL of DAB solution (1 mg ml^{-1} 3-3'-diaminobenzidine-HCl pH 3.8; D8001, Sigma-Aldrich, St. Louis, MO). The samples were kept in the dark overnight and then fixed and de-stained in a 3:1:1 solution of ethanol:lactic acid:glycerol (v/v/v). After clearing, samples were transferred to 60% glycerol for storage until further analysis. For imaging, the tissue was placed into a petri dish containing 70% ethanol and viewed under a stereomicroscope (SteRIO Discovery V20, Carl Zeiss, North York, ON). Plants were scored for the production of ROS as indicated by brown staining.

2.9 β-Glucuronidase staining

Inflorescence apices were stained for β-glucuronidase (GUS) activity as previously described (Khan et al., 2012b). Samples were stored in 70% ethanol and imaged with a stereomicroscope (SteRIO Discovery v20, Carl Zeiss, North York, ON, Canada).

2.10 Statistical analysis

All statistical analysis was completed using R software tools (www.R-project.org/.). For qPCR gene expression experiments, one-way analysis of variance (ANOVAs) were used with a

Tukey's post hoc analysis. Significance is indicated by asterisks where $p < 0.05$(*) and $p < 0.001$ (**).

Table 2.1 List of genes and primers for qRT-PCR

Gene descriptions were obtained from TAIR (https://www.arabidopsis.org/).

Gene Name	Code	Microarray fold-change	Functional Description	Primer Sequence (5'-3') Forward, Reverse
Innate Immunity				
FLAGELLIN-SENSITIVE 2 (FLS2)	AT5G46330	2.11	Receptor for bacterial flagellin	ACTCTCCTCCAGGGGCTAAGGAT AGCTAACAGCTCTCCAGGGATGG
BOTRYTIS-INDUCED KINASE1 (BIK1)	AT2G39660	2.51	Kinase that relays innate immune signal	TAAGCCACTCCCATGGTTTC CGTTGTAGTCCGCATCAAGTA
RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD)	AT5G47910	1.42	Enzyme that produces ROS for innate immune signaling and hypersensitive response	CATGGTATCAAGCTCTACCTCAC AACAGCCACCTTGATCATCTTA
At1g51890	AT1g51890	1.71	Leucine-rich repeat protein kinase	CAGCTGTGAGTTCTCTAGATGG GCGGAAACTTCATCTTGGTATG
CALCIUM DEPENDENT PROTEIN KINASE 28 (CPK28)	AT5G66210	4	Kinase in BIK1 innate immune response pathway	GGAACTTCGAATGCACACGGGG GCAGGGCTTGGTGCTCTCTGTG
WRKY DNA-BINDING PROTEIN 33 (WRKY33)	AT2G38470	4.63	Transcription factor, innate immunity, also regulates JA/SA antagonism	GTGGAAGCAAGACAGTGAGAG TGCACTTGTAGTAGCTTCTTGG
WRKY DNA-BINDING PROTEIN 40 (WRKY40)	AT1G80840	21.11	Transcription factor, innate immunity	GAGAGTAGCTCAACGGATCAAG CATCTTTCACAACGAGGGTAGT
WRKY DNA-BINDING PROTEIN 53 (WRKY53)	AT4G23810	7.36	Transcription factor, innate immunity	CACCAGAGTCAAACCAGCCATTAC CTTTACCATCATCAAGCCCATCGG
CALMODULIN-BINDING PROTEIN60 (CBP60g)	AT5G26920	9.19	Transcriptional activator, positive regulator of innate immunity and SA accumulation	AAGAAGAATTGTCCGAGAGGAG GGCGAGTTTATGAAGCACAG
SAR DEFICIENT1 (SARD1)	AT1G73805	1.78	Direct positive regulator of ICS1 enzyme gene required for SA synthesis.	TCAAGGCGTTGTGGTTTGTG CGTCAACGACGGATAGTTTC
FLG22-INDUCED RECEPTOR-LIKE KINASE (FRK1)	AT2g19190	1.44	Innate immunity marker	GCGCAAGGACTAGAGTATCTTC ATCTGACCGCTTCCTTCAAC
GLUCAN SYNTHASE-LIKE 5 (GSL5)	AT4g03550	NC	Callose synthase gene, induced during innate immunity	GAGTACATTCAGGTCGGGAAG CTGAAGAAATCAAGCCTGTGC
PATHOGENESIS-RELATED PROTEIN 2 (PR2)	AT3G57260	11.55	β-1,3-GLUCANASE gene, pathogenesis-protective function	ATCTCCCTTGCTCGTGAATCTC TCGAGATTTGCGTCGAATAGG
ARABIDOPSIS NDR1/HIN1-LIKE 10 (NHL10)	AT2g35980	2.33	Chloroplast-localized, disease resistance function	GATCGAAGCTCATGCCTACTAC CTAGACTGTCCGGCGTTAAA
Reference genes				
GLYCERALDEHYDE-3-PHOSPHATE (GAPC)	AT3g04120	N/A	N/A	TCAGACTCGAGAAAGCTGCTA GATCAAGTCGACCACACGG
ACTIN1 (ACT1)	AT2g37620	N/A	N/A	CGATGAAGCTCAATCCAAACGA CAGAGTCGAGCACAATACCG
UBIQUITIN10 (UBQ10)	AT4g05320	N/A	N/A	CACACTCCACTTGGTCTTGCGT TGGTCTTTCCGGTGAGAGTCTTCA

CHAPTER 3: RESULTS

3.1 Transcriptome analysis of transgenic plants overexpressing *BOP1* reveals a strong link to plant defense

Transgenic plants that overexpress *BOP1* (*BOP1-OE*) are dwarf with previous analysis linking this phenotype to high constitutive levels of the stress-related hormone JA (Khan *et al.*, 2015). Such defects are also hallmarks of "autoimmune" mutants that constitutively express defense genes (van Wersch *et al.*, 2016). To further examine this connection, I used Gene Ontology (GO) analysis to classify microarray data of differentially expressed genes in *BOP1-OE* stems compared to wild-type (Khan *et al.*, 2015). This analysis showed a strong link to plant defense. Among upregulated genes in *BOP1-OE* plants, 35% sorted to GO terms associated with plant defense (Fig. 3.1). The highest proportion of these defense genes were associated with innate immunity (23%) followed by JA-associated defense pathways (14%) and SA-associated plant pathways (12%) (Fig. 3.1 and Supplemental File 1). The robustness of this GO analysis was independently tested by comparing *BOP1-OE* differentially expressed genes to published microarray data sets chosen as representative of each individual defense pathway. For PTI, a dataset showing transcriptional changes in response to three different PAMPs was used (Wan *et al.*, 2008). For JA-mediated plant defense, a dataset showing transcriptional changes in response to *Botrytis cinerea* infection was used (Windram *et al.*, 2012). For SA-mediated plant defense, a dataset showing transcriptional changes in response to SA treatment was used (Blanco *et al.*, 2009). Further rationale for choosing these studies is given in the Materials and Methods. Consistent with GO analysis, the greatest overlap was seen for the dataset representing PTI (Wan *et al.*, 2008). 59% of genes upregulated by PAMP-treatment were upregulated in *BOP1-OE* plants (Fig. S1 and Supplemental File 2). Comparison of the individual transcriptome datasets for PTI,

JA, and SA showed minimal overlap suggesting that these defense responses are highly specific (Fig. S2). These combined data suggest a strong link between *BOP1* overexpression and innate immunity. Interestingly enough, the expression of *BOP1* was not induced in this PTI dataset or datasets depicted University of Toronto's Bio-Analytic Resource for Plant Biology (www. bar.utoronto.ca) expression profile browser (eFP) for flg22 treatments (Winter *et al.*, 2007) suggesting that BOPs may be subject to post translational regulation in this context

3.2 qPCR validation of microarray data

To verify these microarray data and confirm a correlation with innate immunity, transcripts of "signature" PTI genes in wild-type and mutants were measured by qRT-PCR. A panel of genes was chosen to represent all parts of the PTI signaling pathway from pathogen perception to downstream activation of transcription factors and defense genes. Table 2.1 explains the roles of the different genes that were selected as part of the panel. All of the selected genes were differentially expressed in microarray data of *BOP1-OE* stems except for callose synthase (Table 2.1). Transcripts were measured in wild-type, *BOP1-OE* and *bop1 bop2* stems (Fig. 3.2a) and seedlings (Fig. 3.2b). Consistent with the microarray, *BOP1-OE* stems showed higher transcript levels for the majority of PTI "signature" genes compared to wild-type. In particular, significantly higher transcript levels were recorded for flagellin receptor gene *FLS2* (Zipfel *et al.*, 2004) and co-receptor gene *BIK1* (Lu *et al.*, 2010) which were upregulated 3.0-fold and 4.5-fold, respectively. NADPH oxidase enzyme gene *RBOHD* (Morales *et al.*, 2016) and callose synthase gene *GLS5* (Jacobs *et al.*, 2003) were also significantly upregulated 1.3-fold and 1.8-fold, respectively. Significantly higher transcript levels were also recorded for *SARD1* (up-regulated 3.0-fold) and *CPB60g* (upregulated 2.52-fold) required for SA and pipecolic acid biosynthesis during a defense response (Sun *et al.*, 2015; Sun *et al.*, 2017). Conversely, the abundance of *PTI* gene transcripts in

bop1 bop2 stems were the same or reduced compared to wild-type with one exception. Transcription factor gene *WRKY40* is a negative regulator of PTI involved in growth-defense trade-off (Lozano-Durán *et al.*, 2015). This transcript showed little or no change in *BOP1-OE* stems conflicting with the microarray (Table 2.1) but was significantly upregulated 3.3-fold in *bop1 bop2* stems suggesting possible disruption in growth-defense balancing mechanisms. In general, similar trends were observed in both stems and seedlings indicating that results are not strongly tissue-specific (Fig. 3.2ab). Thus, *BOP1* overexpressing plants have elevated constitutive levels of PTI-associated defense genes compared to wild-type suggesting a positive role in plant immunity.

3.3 BOP1/2 and TGA1/4 co-regulate a subset of defense genes

Clade I TGA1 and TGA4 are essential co-factors for BOP-dependent regulation of plant development. BOPs bind to clade I TGAs for recruitment to DNA resulting in gene activation (Wang *et al.*, 2018). Clade I TGAs were previously characterized as having a role in innate immunity (Kesarwani *et al.*, 2007). Transcriptome studies of the *tga1 tga4* mutant uncovered defects in apoplastic defense mechanisms important for the PTI response (Shearer *et al.*, 2012; Wang & Fobert, 2013). Clade I TGAs are also transcriptional promotors of *SARD1* and *CBP60g* transcription factor genes required for pathogen-induced SA biosynthesis (Sun *et al.*, 2017). These data prompted us to test if BOPs and clade I TGAs co-regulate a subset of defense genes important for innate immunity. The induction of *SARD1*, *CBP60g*, *FLS2*, *RBOHD*, and *PR2* genes was measured in seedlings of wild-type and mutants: *BOP1-OE*, *bop1 bop2, tga1 tga4*, *bop1 bop2 tga1 tga4* using *flg2* as a negative control (Fig. 3.4). As expected, all of these genes were significantly induced in wild-type after 4 hours of *flg22* treatment and no induction was observed in *fls2* mutants which do not perceive flagellin (Zipfel *et al.*, 2004; Wang *et al.*, 2009; Wang *et al.*, 2011; Morales *et al.*, 2016). The 4 hour time point was used as a conservative time point where early as well as

later transcriptional changes would be detected. I also collected samples from a 12 hour time point in case I saw little to no induction in these defense genes after 4 hours but this data was not analyzed. Consistent with microarray data and Fig. 3.2, all genes were expressed at significantly high levels in *BOP1-OE* transgenic lines in the absence of elicitor. These levels were comparable to wild-type plants after flg22 treatment. Interestingly, the most statistically significant gene induction by flg22 treatment was observed in *BOP1-OE* mutants. By contrast, induction was impaired in *bop1 bop2, tga1 tga4,* and *bop1 bop2 tga1 tga4* loss-of-function mutants. These data suggest that BOP1/2 and clade I TGAs contribute to the activation of PTI genes in response to flg22 elicitor.

3.3 *BOP1-OE* plants prioritize defense over development

Initiation of a defense response triggers a decrease in growth rate since resources are diverted to combat pathogen invasion (Huot *et al.*, 2014). We therefore used seedling growth inhibition assays (Schwessinger *et al.*, 2011; Monaghan *et al.*, 2014) to measure this response in wild-type and mutants: *BOP1-OE*, *bop1 bop2*, *tga1 tga4*, *bop1 bop2 tga1 tga4* using *fls2* as a negative control. Seedlings were grown on plates for 6 days and then transferred into liquid media with or without flg22 elicitor. Seedlings were allowed to grow for an additional 10 days and then the average fresh weight of flg22-treated seedlings was compared to mock-treated seedlings and expressed as a ratio. Fig. 3.4 shows that growth of wild-type seedlings was reduced by 78% in presence of elicitor. This inhibition was even more pronounced in plants overexpressing *BOP1* where the addition of flg22 resulted a growth reduction of 94.8% compared to mock-treated seedlings. By contrast, *bop1 bop2* and *tga1 tga4* double mutants showed a diminished growth inhibition response compared to wild-type. The quadruple *bop1 bop2 tga1 tga4* mutant showed the smallest growth inhibition response to flg22 treatment with a growth reduction of 39.9%

compared to mock-treated control seedlings. Little or no growth inhibition was observed in *fls2* mutants since a functional receptor is required to perceive flg22. These collective data support a positive role for BOPs and clade I TGAs in regulating growth-defense trade-offs in innate immunity.

3.5 ROS and callose are ectopically deposited in *BOP1-OE* seedlings

Transgenic *BOP1-OE* plants have a heightened growth inhibition response to PAMP and display constitutive expression of defense genes, including *RBOHD* required for ROS production and *GSL5* required for synthesis of callose—hallmarks of a PTI response. Many auto-immune mutants display higher constitutive levels of ROS and callose because defense pathways are active (van Wersch *et al.*, 2016)

To test for constitutive ROS accumulation, 10-day-old seedlings of wild-type and mutants: *BOP1-OE* and *bop1 bop2* were stained with diaminobenzidine (DAB) which detects the presence of H_2O_2 (Thordal-Christensen *et al.*, 1997). Fig. 3.4 shows that while wild-type and *bop1bop2* seedlings displayed little or no DAB staining, *BOP1-OE* seedling leaves frequently displayed dark brown staining indicative of H_20_2 presence. To test for constitutive callose depositions, 10-day-old seedlings of wild-type and mutants *BOP1-OE* and *bop1 bop2* were stained with aniline blue and viewed under UV light to observe callose. Fig. 3.5 shows that wild-type and *bop1 bop2* seedling leaves displayed little or no callose staining whereas a large majority of *BOP1-OE* leaves had callose deposits. These collective data provide evidence that ROS and callose deposition is upregulated in *BOP1-OE* mutants consistent with an auto-immune phenotype.

3.6 GO analysis of BOP1-OE and AZ transcripts centers on plant defense terms

Abscission is a natural process in plants that allows organs to detach in response to developmental cues or stress signals (Patharkar & Walker, 2017). Exposed cells on the plant body

after abscission secrete a protective layer of cuticle that resists water loss and pathogen attack (Lee *et al.*, 2018). Transcriptome studies have identified numerous differentially-regulated "defense" genes encoding NADPH oxidases, peroxidases, and *PR* proteins in AZs although the role of these factors is debated (Meir *et al.*, 2011; Kim *et al.*, 2015; Lee *et al.*, 2018). Given that BOPs are required for AZ differentiation and possibly plant defense, up-regulated genes in the *BOP1-OE* microarray dataset were compared to receptacle AZ transcriptome data (Lee *et al.*, 2018). This AZ data set contained 8605 upregulated genes of which 1057 matched to upregulated genes in the *BOP1-OE* dataset. GO annotation of these 1057 overlapping genes showed that the most significant categories, represented by red and orange colour, were terms associated with plant defense (Fig. 3.7).

3.7 Callose deposition in abscission zones

BOP1/2 were recently shown to regulate the production of ROS in the AZ needed for downstream polymerization of lignin required for separation (Lee *et al.*, 2018). ROS signaling during PTI induces callose at sites of infection (Daudi *et al.*, 2012). To test if callose deposition is changed by loss or gain of BOP function in receptacle AZs, staining was carried out in wild-type and mutants. Fruits were sampled from approximately position 6 where abscission takes place until maturity. Floral organs on *bop1 bop2* fruits that do not abscise naturally were removed to expose the AZ. Little or no callose was detected in the AZ of WT and *bop1 bop2* mutants. By contrast, a majority of *BOP1-OE* fruits had callose deposits in the receptacle AZ. These collective data support the hypothesis that BOP1/2 roles in PTI defense may overlap with the abscission process.

3.8 *PR* gene expression in abscission zones

To further assess the expression of defense marker genes in the abscission zones, we stained transgenic plants expressing a *PR1:GUS* reporter gene (Shapiro & Zhang, 2001). *PR1*,

PR2, and *PR5* are group of genes encoding secreted defense proteins (van Loon *et al.*, 2006). These genes are upregulated in our *BOP1-OE* microarray dataset. In particular, *PR1* and *PR2* show a 31.3-fold and 11.55-fold increase, respectively. Fig. 3.9 shows that *PR1* is expressed dominantly in the abscission zone of the siliques. What's more interesting is the expression pattern appears to be developmentally regulated, since the expression seems to peak during abscission (position 6 or so) and then decrease post-abscission (positions 8 and later) when a protective layer of cuticle is formed. These data suggest that defense genes may be transiently activated in the AZ, during active shedding of floral organs as a possible resistance mechanism against pathogens or for another reason.

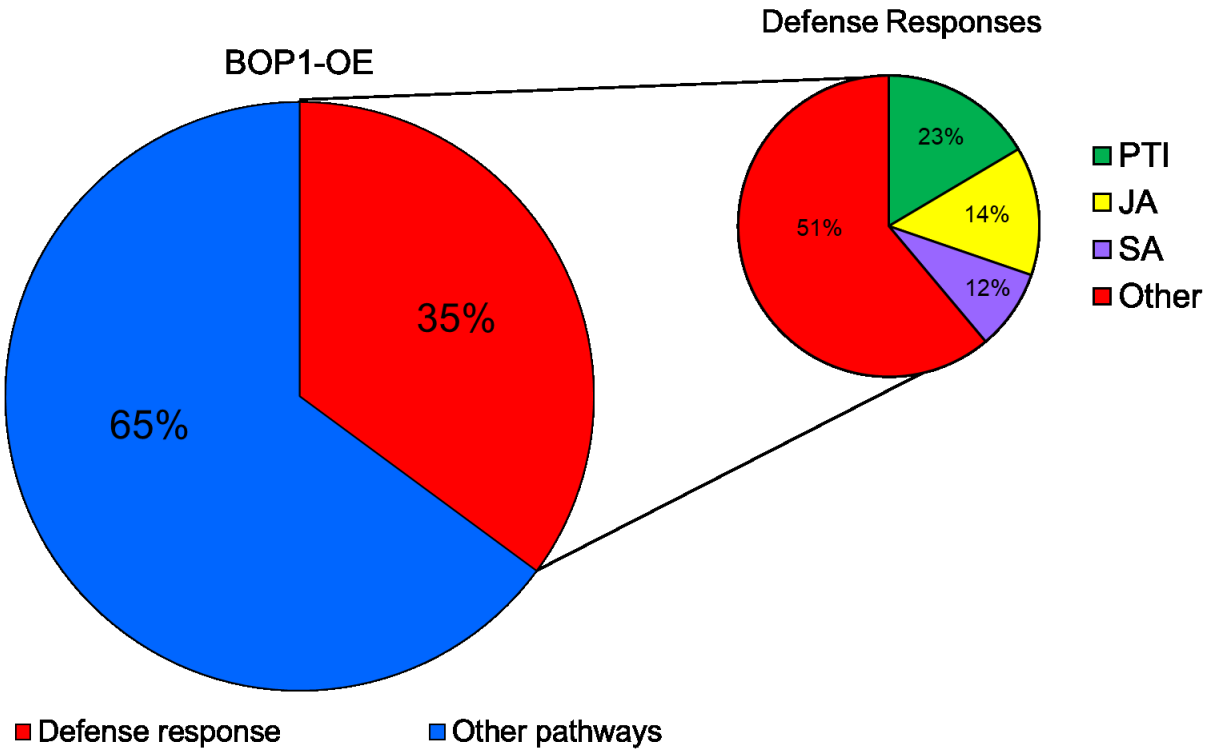

Figure 3.1 Pie chart showing GO analysis of *BOP1-OE* microarray dataset.

(Left) 1044 of 2974 differentially upregulated genes in stems of *BOP1-OE* transgenic plants sorted to GO terms related to plant defense (Right) Defense genes in *BOP1-OE* were sorted into different defense categories as follows: 239 PTI-associated genes; 144 JA pathway genes and 122 SA pathway defense genes. Data were sorted using agriGO and Princeton web-based tools against *Arabidopsis thaliana* TAIR10 genome annotation as described in the Materials and Methods. See Supplemental File 1 for list of genes in each category. During the GO analysis, 180 genes were not assigned to a category and were therefore left out of the analysis.

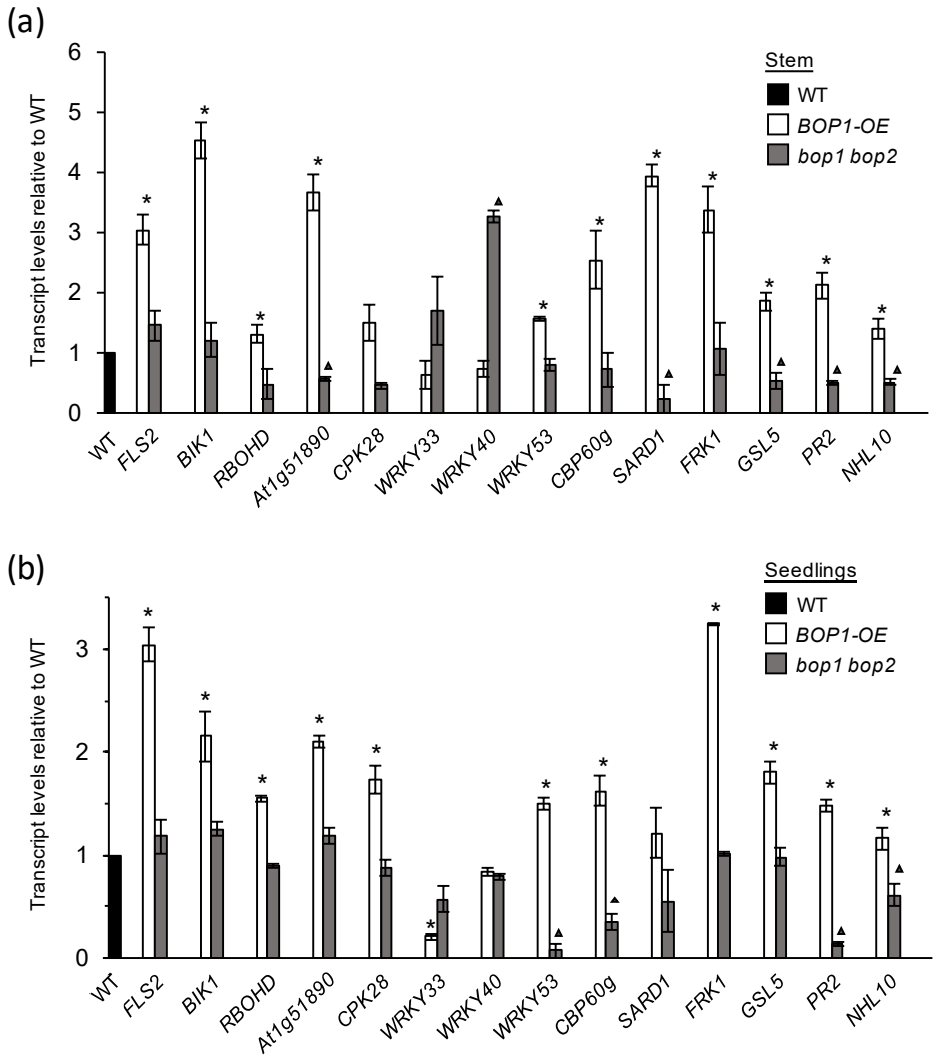

Figure 3.2 Upregulation of PTI-associated defense genes in *BOP1-OE* transgenic plants confirmed by qRT-PCR.

Transcript level of genes characteristic of a PTI defense response were monitored in WT, *bop1 bop2,* and *BOP1-OE* plants. For a description of genes, see Table 2.1. Values were normalized to WT transcript levels in (a) stems and (b) seedlings. Data represent the mean ± SD of three biological replicates. Reference genes used for normalization were *GAPC* and *ACT1.* Asterisks, significantly upregulated and triangles, significantly downregulated relative to WT ($p \leq 0.05$, one way ANOVA with Tukey's post-hoc test).

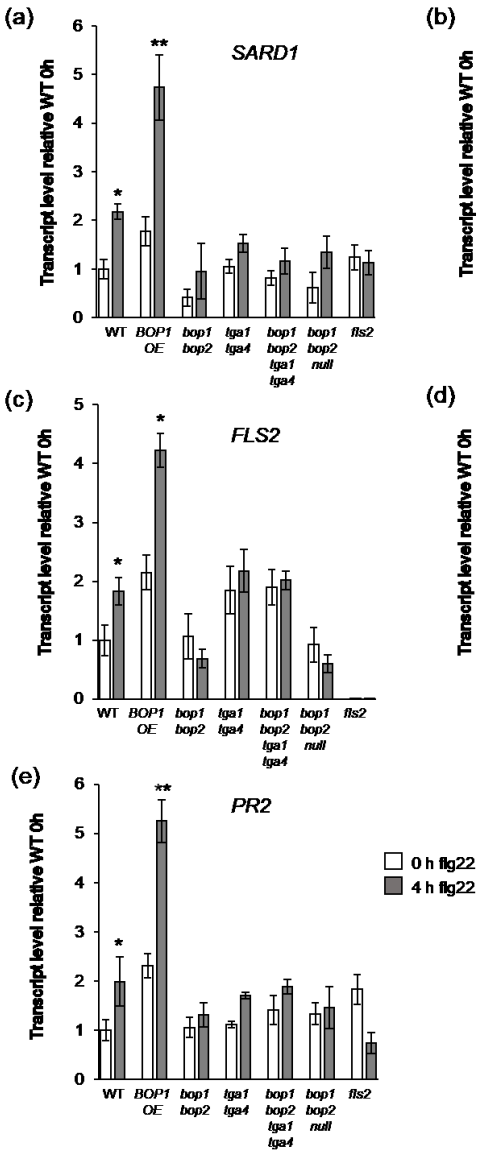

Figure 3.3 BOPs and Clade I TGAs co-regulate a subset of PTI genes.

Relative transcript levels of selected PTI defense genes at 0 hours and 4 hours following treatment with 1 μm flg22 elicitor. Values are shown relative to WT 0 h. Data represent the mean ± SD of three biological replicates normalized to the reference genes *UBQ10* and *ACTIN1*. Each replicate contained 8-12 seedlings. Asterisks, represent significantly different values between 0 and 4 hour in each genotype (*$p \leq 0.05$, **$p \leq 0.0001$; one-way ANOVA with Tukey's post-hoc test).

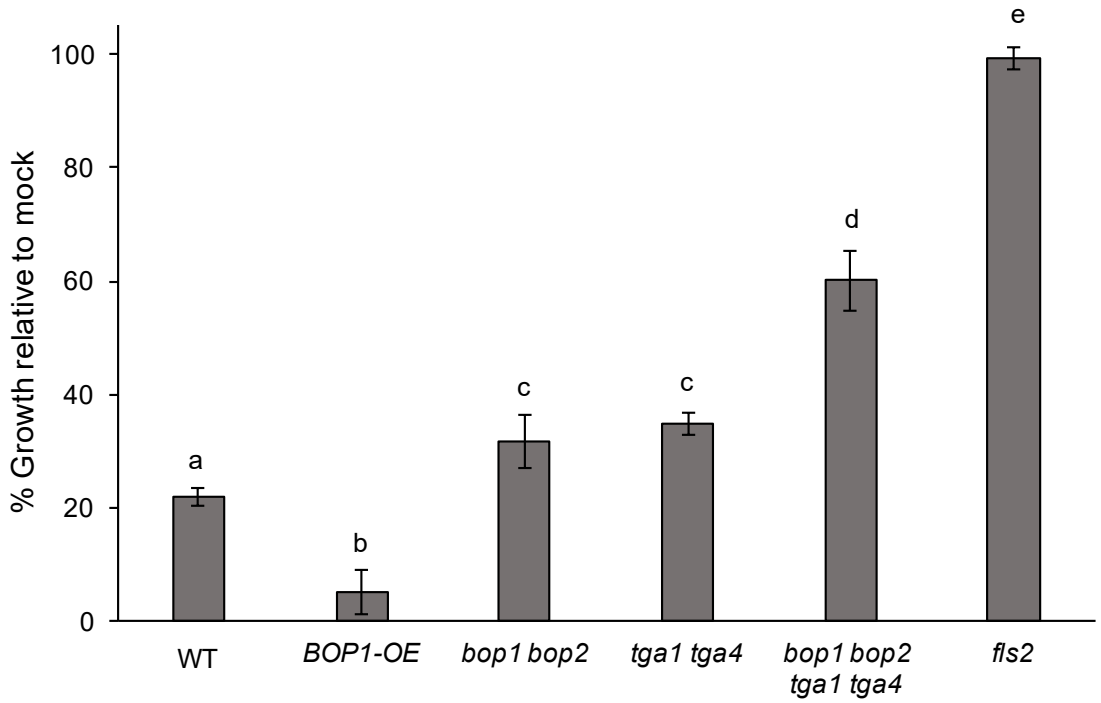

Figure 3.4 PAMP-triggered seedling growth inhibition response of wild-type and mutants

Quantification of seedling growth inhibition for wild-type and mutants exposed to 1 μm flg22. Data show percent growth of flg22-treated seedlings versus mock. Data represent mean ± SD for 3 biological replicates except *fls2* negative control, one replicate. Each replicate contained $n \geq 10$ seedlings. Lowercase letters represent significantly different groups ($p \leq 0.001$, one-way ANOVA with Tukey's post-hoc test)

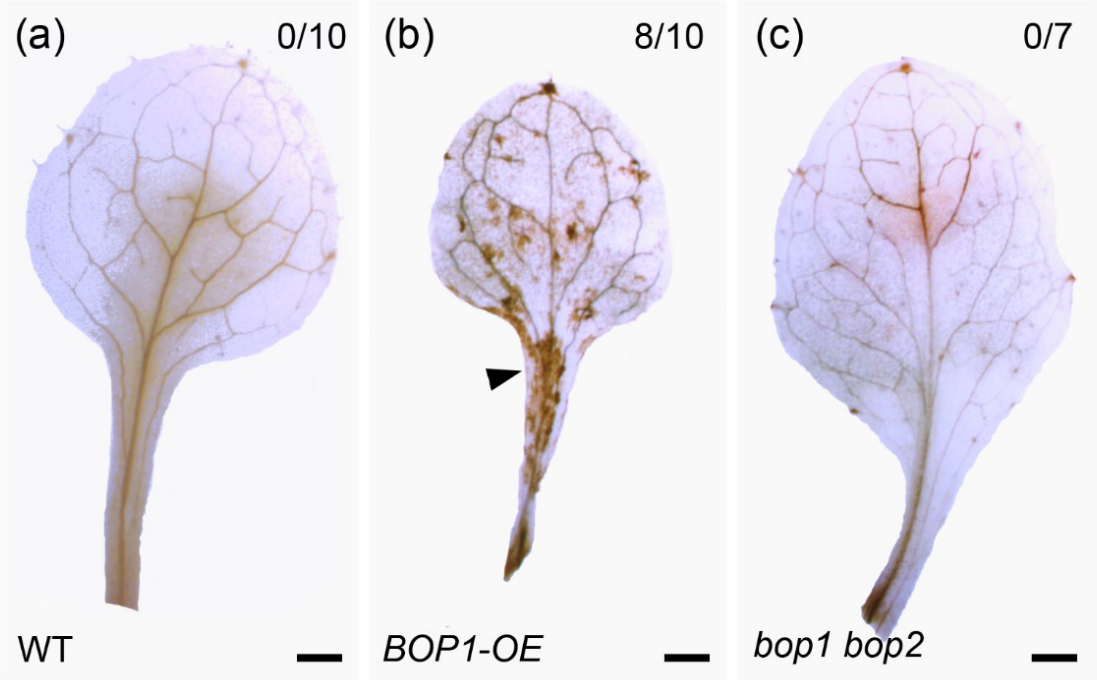

Figure 3.5 DAB-stained wild-type, *BOP1-OE*, and *bop1 bop2* leaves.

Representative images of rosette leaves from 10-day-old seedlings. Top right of panels, number of leaves with significant ROS staining in test population. (a) WT showing little or no ROS staining. (b) *BOP1-OE* showing abundant ROS staining (arrow). (c) *bop1 bop2* showing little or no ROS staining, similar to WT. Scale bar, 0.5 mm.

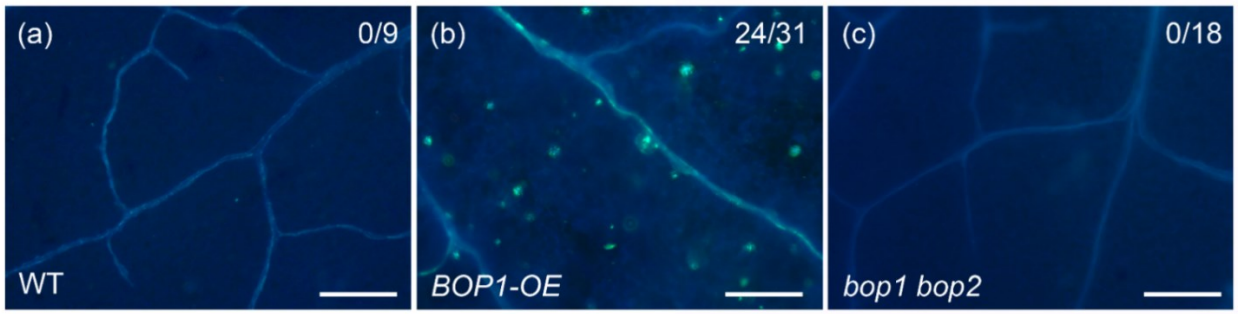

Figure 3.6 Aniline-blue stained WT, *BOP1-OE*, and *bop1 bop2* leaves showing callose deposition.

Representative images are shown for 10-day-old seedlings. Top right of panels, number of callose-positive leaves in test population. (a) WT showing little or no callose staining. (b) *BOP1-OE* showing abundant callose staining. (c) *bop1 bop2* showing little or no callose staining, similar to WT. Scale bars, 200 μm.

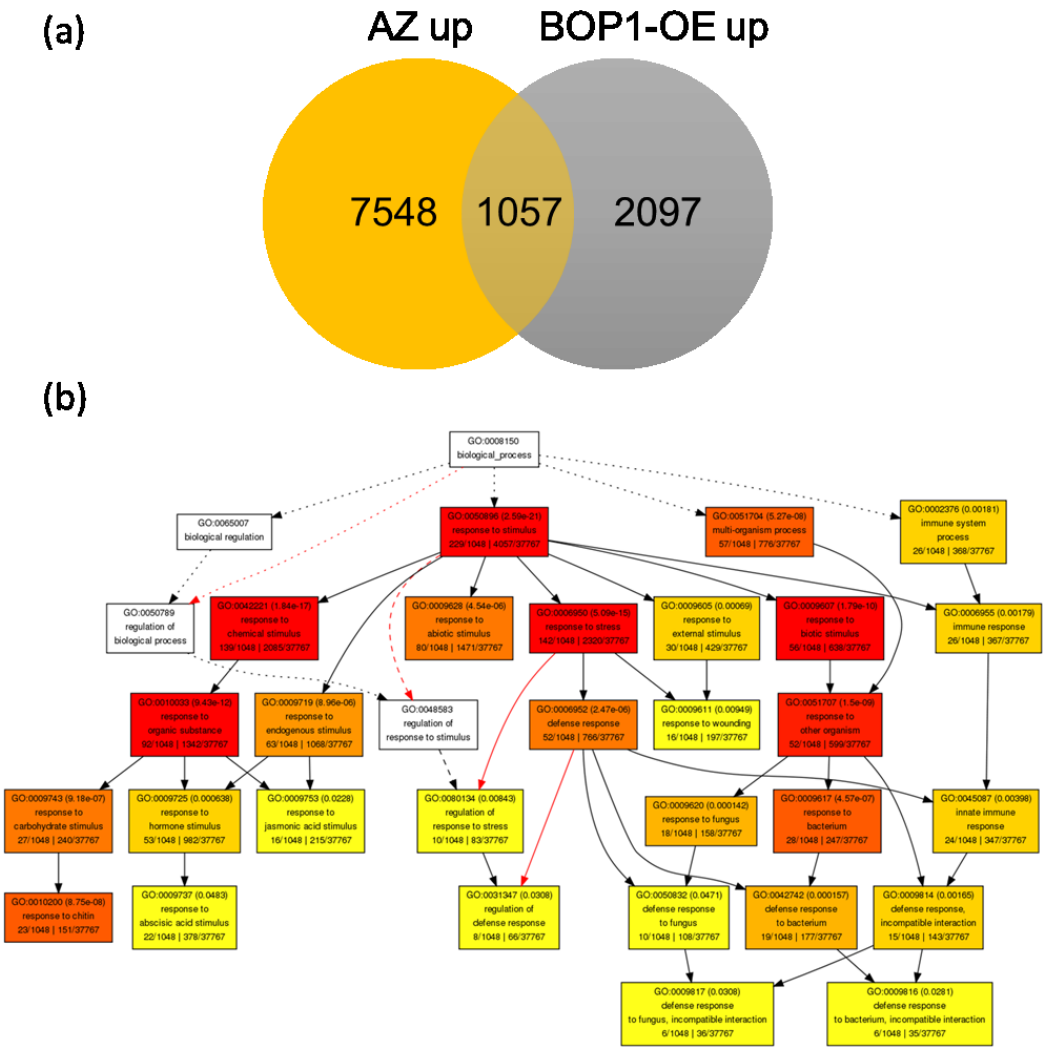

Figure 3.7 Venn diagram and GO analysis showing overlap of genes expressed in AZs compared to BOP1-OE microarray.

(a) Receptacle AZ dataset (Lee *et al.*, 2018) versus *BOP1-OE* microarray dataset of upregulated genes showing an overlap of 1057 genes (33.5% of *BOP1-OE* dataset). Genes were sorted using GeneVenn's online tool (Pirooznia *et al.*, 2007). (b) GO ontology analysis of overlap showing strong alignment with plant defense terms (red, greatest number of terms and white, least number of terms). Image created using agriGO v2.0 online tool. After converting to GO terms, 9 genes were uncategorized and therefore left out of the analysis.

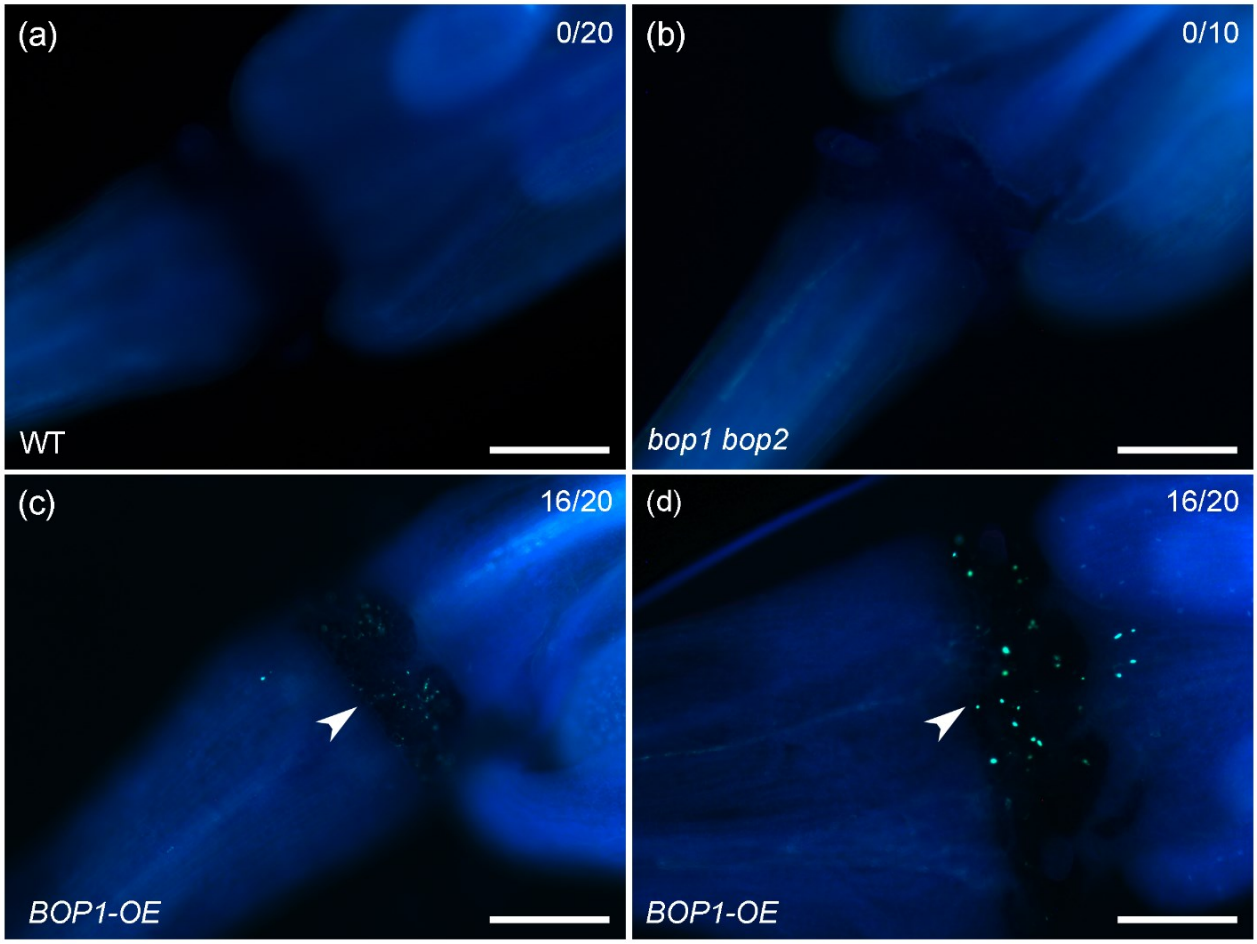

Figure 3.8 Aniline-blue stained WT, *bop1 bop2*, and *BOP1-OE* flower receptacles showing callose deposition in AZs.

Representative images are shown for post-abscission stages of fruit development. Callose deposits appear as fluorescent punctate spots under UV light. Top right of panels, number of callose-positive AZs in test population. (a) Wild-type showing no callose deposition in AZ. (b) *bop1 bop2* with floral organs removed showing no callose deposition in AZ. (c) *BOP1-OE* showing abundant callose deposits in the AZ (arrow). (d) *BOP1-OE* close-up of AZ region showing callose deposits (arrow). Scale bars, 200 µm except 100 µm in (d).

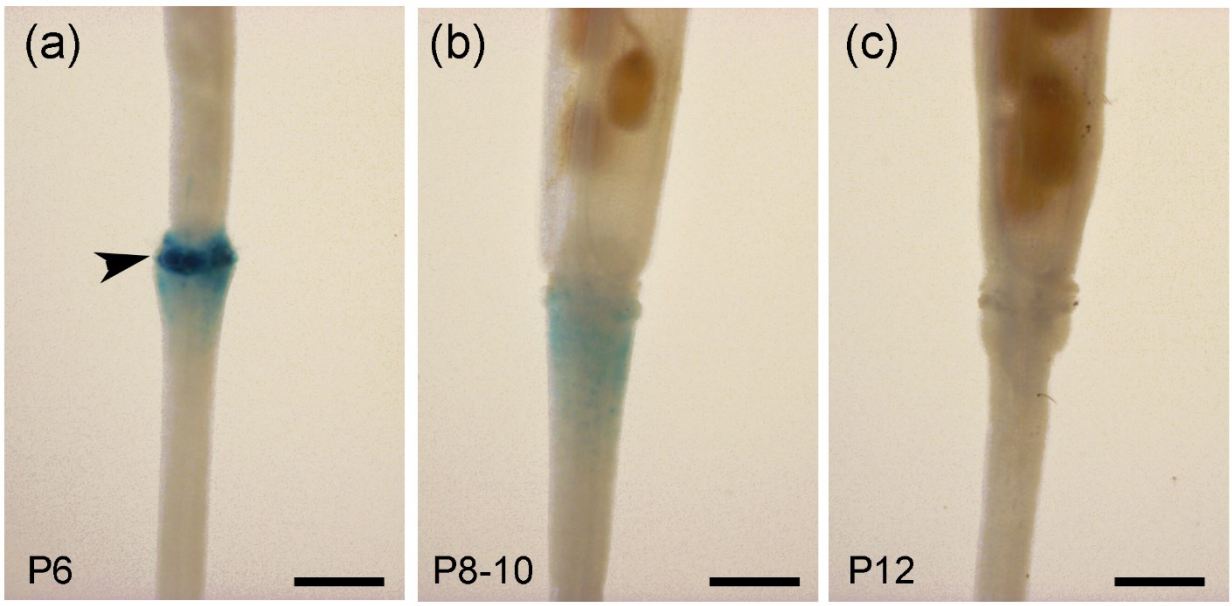

Figure 3.9 Detection of *PR1:GUS* defense gene expression in the AZ of fruits.

Representative images are shown for developing fruits at different stages of abscission. The youngest flower on the inflorescence showing white petals is defined as position 1 (Bleeker & Patterson, 1997). In wild-type, abscission takes place at positions 4-6 on the inflorescence. No staining was observed at positions where organs were still attached. (a) Position 6, during or shortly after abscission. (b) Position 8-10, post-abscission. (c) Position 12, late post-abscission. Scale bars, 0.5 mm

CHAPTER 4: DISCUSSION

Plants are the target of a broad spectrum of pathogens that include insects, fungi and bacteria (Jones & Dangl, 2006). In order for plants to properly detect and combat these invading pathogens, plants use regulated mechanisms of defense that are both innate and induced (Jones & Dangl, 2006). Defense responses are imperative for plant survival, however they are resource-demanding and often come at the expense of plant growth (Huot *et al.*, 2014).

BTB-ankyrin proteins are an evolutionarily conserved group of co-transcriptional activators in the plant kingdom (Khan *et al.*, 2014). NPR1-like and BOP-like BTB-ankyrin proteins are found in liverworts (Fig 1.4) and simple mosses (Saleh *et al.*, 2011). This subdivision is preserved in flowering plants where NPR1-like members perceive salicylic acid to activate SAR and BOP-like members regulate plant development (Khan *et al.*, 2014). Using transcriptome analysis of *BOP1* transgenic overexpressing plants, I identified and partially characterized a previously undiscovered role for BOPs in plant innate immunity. Plants constitutively expressing *BOP1* have high basal levels of innate defense genes and exhibit hallmarks of autoimmunity (van Wersch *et al.*, 2016). Conversely, *bop1 bop2* loss-of-function mutants have impaired induction of defense genes. These collective data indicate role for BOPs in innate immunity. At least a subset of defense genes may be activated in conjunction with clade I TGAs, which we have recently shown are required for BOP-dependent regulation of plant development.

4.1 *BOP1-OE* transcriptome reveals a strong link to plant defense.

Transgenic plants overexpressing *BOP1* or *BOP2* have stunted growth coupled with upregulated transcripts involved in JA and SA signaling pathways suggesting defects in growth-defense balance (Khan *et al.*, 2015). To explore these findings in more detail, microarray data of differentially expressed genes in *BOP1-OE* stems was further analyzed. GO analysis revealed that

about one third of upregulated genes (1057 of 2974) were related to plant defense. Further classification showed that these genes were not specific to any one pathway but included a large component associated with PTI defenses (22%). Over representation of PTI defense genes was also observed when comparing *BOP1-OE* differentially expressed genes with datasets representative of PTI, JA, and SA defense responses. To test these data, the transcript levels of characteristic PTI genes were directly monitored in WT, *BOP1-OE*, and *bop1 bop2* plants using qRT-PCR. Sure enough, a majority of PTI genes in *BOP1-OE* internodes and seedlings were upregulated relative to wild-type in agreement with the microarray. Among these upregulated genes were *SARD1* and *CBP60g* required for pathogen-induced SA and pipecolic acid biosynthesis (Zhang *et al.*, 2010). Also upregulated were the NADPH oxidase gene *RBOHD* which produces ROS (Morales *et al.*, 2016), pathogen-induced callose synthase gene *GLS5* (Jacobs *et al.*, 2003) and β-1,3-glucanase gene *PR2*, also involved in pathogen-induced callose deposition (Oide *et al.*, 2013). In accord, *BOP1-OE* seedling leaves exhibited high resting levels of ROS staining and abundant callose deposits compared to wild-type. *BOP1-OE* seedlings treated with flg22 elicitor also showed a hypersensitive growth inhibition response compared to wild-type. All of these characterizations of *BOP1-OE* fit the definition of autoimmunity (van Wersch *et al.*, 2016)

4.2 BOPs and TGAs co-regulate a subset of PTI genes

BOP1/2 activity in plant development depends on clade I TGA factors for recruitment to DNA (Wang *et al.*, 2018) raising the possibility that similar interactions are required for BOP-dependent immune functions. Clade I TGAs interact with NPR1 in SA-treated leaves but this interaction is not critical for SAR (Després *et al.*, 2003). Instead, clade I TGAs play a more significant role in innate immunity since knockout mutants are impaired in PTI defenses against *Pseudomonas syringae* (Kesarwani *et al.*, 2007; Wang & Fobert, 2013; Sun *et al.*, 2017). Defects

in this pathway are due in part to ER-related secretion defects in *tga1 tga4* mutants that impair cell wall-based extracellular defenses (Wang & Fobert, 2013). Western blots show that *tga1 tga4* mutants accumulate lower levels of PR1 protein in the apoplast following treatment with flg22 despite having normal levels of *PR1* transcript (Wang & Fobert, 2013). Clade I TGAs are also required for the full induction of *SARD1* and *CBP60g* genes required for pathogen-induced SA and pipecolic acid biosynthesis (Sun *et al.*, 2017). ChIP assays demonstrated direct binding of TGA1 to the *SARD1* promoter (Sun *et al.*, 2017). In my study, induction of *SARD1* and *CBP60g* along with several other genes was impaired in *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* mutants suggesting that BOPs may directly regulate a subset of PTI genes using clade I TGAs for recruitment to DNA. Future experiments using ChIP will allow us to test if characteristic PTI genes like *SARD1* are direct targets of BOPs and TGAs. ChIP assays can also be used to test if BOP recruitment to the promoter of defense genes is enhanced by PTI signals.

Characterization of NPR1 interaction with TGA2 during SAR provides clues about how clade I TGAs might contribute to BOP-dependent transcriptional regulation of defense genes. Biochemical studies on the *PR1* promoter show that TGA2 is a repressor under normal conditions (Rochon *et al.*, 2006). During a defense response, SA binds to NPR1 causing a conformational change that enhances its function by releasing a C-terminal transactivation domain from inhibition (Wu *et al.*, 2012). TGA2 is converted from a repressor into an activator by incorporation of NPR1 into the complex (Rochon *et al.*, 2006; Boyle *et al.*, 2009). Clade I TGAs might likewise function as repressors until the addition of BOP1. Constitutive elevation of defense genes in *tga1 tga4* mutants has been reported (Lindermayr *et al.*, 2010; Shearer *et al.*, 2012) but no such basal elevation of defense genes was detected in my study with the possible exception of *FLS2* (Fig. 3.3).

4.3 Direct pathogen studies support role for BOPs in innate immunity

Pathogen testing was carried out to directly test the role of BOPs in plant innate defense. These experiments were performed by Bodunde Oyetoran in collaboration with Dr. Myron Smith at Carleton University. In brief, wild-type and mutants: *BOP1-OE*, *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* were directly challenged with a *hrcC*$^{-}$ mutant of *Pseudomonas syringae* that lacks the machinery to deliver effectors into plant cells (Hauck *et al.*, 2003). As a result, PTI is the predominant immune response limiting bacterial growth and disease symptoms against this mutant (Hauck *et al.*, 2003). Bacterial titres of *HrcC*$^{-}$ mutant were measured in infiltrated leaves of four-week-old plants. Four days after infection, leaves of *BOP1-OE* plants harbored significantly lower titres of bacteria compared to wild-type indicating resistance. By contrast, leaves of *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* plants harboured significantly higher titres of bacteria compared to wild-type indicating sensitivity. Bacterial growth among *bop1 bop2, tga1 tga4*, and *bop1 bop2 tga1 tga4* mutants was similar. A transient burst of ROS 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. No ROS reduction was observed in *bop1 bop2* mutants. 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 collective results strongly support a role BOPs in innate plant defense, possibly in the same genetic pathway was clade I TGAs.

4.2 Growth-defense trade-off

Plants that are geared to implementing costly defense responses are continuously re-allocating their resources at the expense of normal development. Studies have identified an

important "growth-defense trade-off" that encompasses cross-talk between many signalling hormones (Huot et al. 2014). For example, JA antagonizes growth by inhibiting multiple steps of the gibberellin signaling cascade: by inhibiting gibberellin biosynthesis (Magome *et al.*, 2004; Heinrich *et al.*, 2012), by stabilizing DELLA repressors of gibberellin signaling (Yang *et al.*, 2012) and by inducing AP2/ERF transcription factors that repress growth (Magome *et al.*, 2008; Sun *et al.*, 2008; Kang *et al.*, 2011; Licausi *et al.*, 2013). BOP1-OE related growth defects are previously shown to correlate with an increase in JA biosynthesis resulting in higher steady state levels of hormone in apices and stems (Khan *et al.*, 2015). However, inactivation of a JA biosynthetic enzyme in BOP1-OE transgenics did restore normal growth suggesting that defects in this mutant are complex (Khan *et al.*, 2015). A recent chemical biology screen identified BOP1 as a dominant negative inhibitor of brassinosteroid signaling by binding to BZR1, a master regulator of brassinosteroid responses including growth (Huot *et al.*, 2014; Shimada *et al.*, 2015). Perhaps PTI signals enhance BOP1/2 activity leading to an inhibition of BR signalling and reduction in growth. BR signaling inhibits PTI responses include ROS production but evidence for mutual repression is sketchy (Lozano-Durán & Zipfel, 2015). BOP1/2 interactions with BZR1 during PTI is an important area for future study. Seedling growth inhibition assays support a role for BOPs in controlling growth defense trade-off. *BOP1-OE* plants treated with elicitor exhibited a 94.8% reduction in growth compared to 78% reduction in growth for wild-type. Conversely, growth inhibition in *bop1 bop2* and *tga1 tga4* mutants was diminished compared to wild-type and further diminished in *bop1 bop tga1 tga4* quadruple mutants suggesting an additive response. Besides functioning as a transcriptional co-factor, BOP1/2 proteins are substrate adaptors for CULLIN3-based E3 ubiquitin ligase complexes involved in protein degradation (Zhang *et al.*, 2017; Chahtane

et al., 2018). This secondary role of BOP1/2 may account for additive functions of BOP1/2 and clade I TGAs in the seedling growth inhibition assay.

4.6 Defense link to abscission

BOPs are strongly expressed at abscission in the plant where are required for AZ differentiation and contribute to organ separation (McKim *et al.*, 2008; Corrigan, 2018; Lee *et al.*, 2018). Conceptual overlaps between innate immunity and abscission have yet to be explored in detail. Both pathways rely on the recognition of signals through similar receptors and similar *BAK1/SERK* co-receptors and converge onto similar MAPK signalling cascades to induce an overlapping subset of genes involved in cell wall modification and reinforcement (Meir *et al.*, 2011; Estornell *et al.*, 2013; Kim *et al.*, 2015; Patharkar & Walker, 2017). My data show that approximately 33.5% of *BOP1-OE* upregulated genes overlap with enriched transcripts present in AZ. Classification of these overlapping genes shows an enrichment of terms associated with defense responses. Among these, cell-wall associated NADPH oxidases and peroxidases are an interesting group because they play an important role in ROS production (O'Brien *et al.*, 2012). Extracellular ROS has direct antimicrobial activity as well as plays roles in cell signaling for the induction of defense genes, cell-wall protein cross-linking, lignin polymerization, and callose deposition (Ralph *et al.*, 2004; Daudi *et al.*, 2012; O'Brien *et al.*, 2012). ROS are also produced in abscission zones where their production is dramatically reduced in *bop1 bop2* mutants, possibly contributing to a missing layer of lignified layer of cells at the base of organs that guides the separation process (Lee *et al.*, 2018). BOP1/2 may directly contribute to ROS production in AZs given that *RBOHD* transcript and ROS staining in *BOP1-OE* leaves is elevated under resting conditions. Callose deposition is also increased in *BOP1-OE* AZs but there is no known role for

callose in abscission and this is supported by lack of induced expression of callose genes in AZ datasets (Kim *et al.*, 2015; Lee *et al.*, 2018).

4.8 Defense markers are expressed in the abscission zones

Another way to assess the link between the abscission process and defense response is to look at the expression patterns of marker genes throughout the abscission process. *PR1:GUS* expression pattern is only seen in the abscission zone area of fruits during the active separation of organs. Diminished expression of *PR1:GUS* during the post-abscission phase suggests that its activity is no longer required. This diminished expression following separation correlates with formation of a protective layer of cuticle over the scar (Lee *et al.*, 2018). Future work will examine the pattern of other defense marker genes in abscission zones. Depending on their timing of expression in the abscission zone, they may be directly required for the separation process or protect exposed cells from pathogen attack while the cuticle layer is being formed.

4.9 Conclusions and future directions

In summary, my thesis unveils a previously undiscovered role for BOP1/2 in plant innate immunity. My work provides evidence that BTB-ankyrin proteins from both subclades contribute to plant defense. Future work will examine how interactions between BOP1/2 and NPR1 subclade members possibly control defense outcomes. Preliminary evidence from my study suggests that BOPs interact with clade I TGAs to regulate the transcription of genes involved both in defense and development. Future work will investigate this prediction as well as the role innate immunity genes in the abscission process. Finally, characterization of the autoimmune phenotype of BOP1-OE transgenic lines is a unique tool for dissecting growth-defense trade-off mechanisms in plants. Understanding these mechanisms can provide a more optimal balance between productivity and disease resistance in crop plants.

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SUPPLEMENTAL MATERIALS

Gene Name	Genotype	Experimental p-value	
		Stem	Seedling
FLS2	*BOP1-OE*	0.0455	0.0409
	bop1 bop2	0.0483	0.9987
BIK1	*BOP1-OE*	0.0370	0.0484
	bop1 bop2	0.6216	0.0842
RBOHD	*BOP1-OE*	0.0507	0.0460
	bop1 bop2	0.0588	0.0908
At1g51890	*BOP1-OE*	0.0390	0.0296
	bop1 bop2	0.0530	0.0587
CPK28	*BOP1-OE*	0.0880	0.0550
	bop1 bop2	0.0603	0.6460
WRKY33	*BOP1-OE*	0.0668	0.0003
	bop1 bop2	0.0787	0.0678
WRKY40	*BOP1-OE*	0.0544	0.4394
	bop1 bop2	0.0265	0.2374
WRKY53	*BOP1-OE*	0.0390	0.0210
	bop1 bop2	0.3704	0.0235
CBP60g	*BOP1-OE*	0.0402	0.0210
	bop1 bop2	0.0626	0.0402
SARD1	*BOP1-OE*	0.0185	0.0679
	bop1 bop2	0.0520	0.0766
FRK1	*BOP1-OE*	0.0348	0.0006
	bop1 bop2	0.9987	0.2301
GSL5	*BOP1-OE*	0.0460	0.0280
	bop1 bop2	0.0523	0.4840
PR2	*BOP1-OE*	0.0320	0.0340
	bop1 bop2	0.0535	0.0277
NHL10	*BOP1-OE*	0.0451	0.0678
	bop1 bop2	0.0456	0.0320

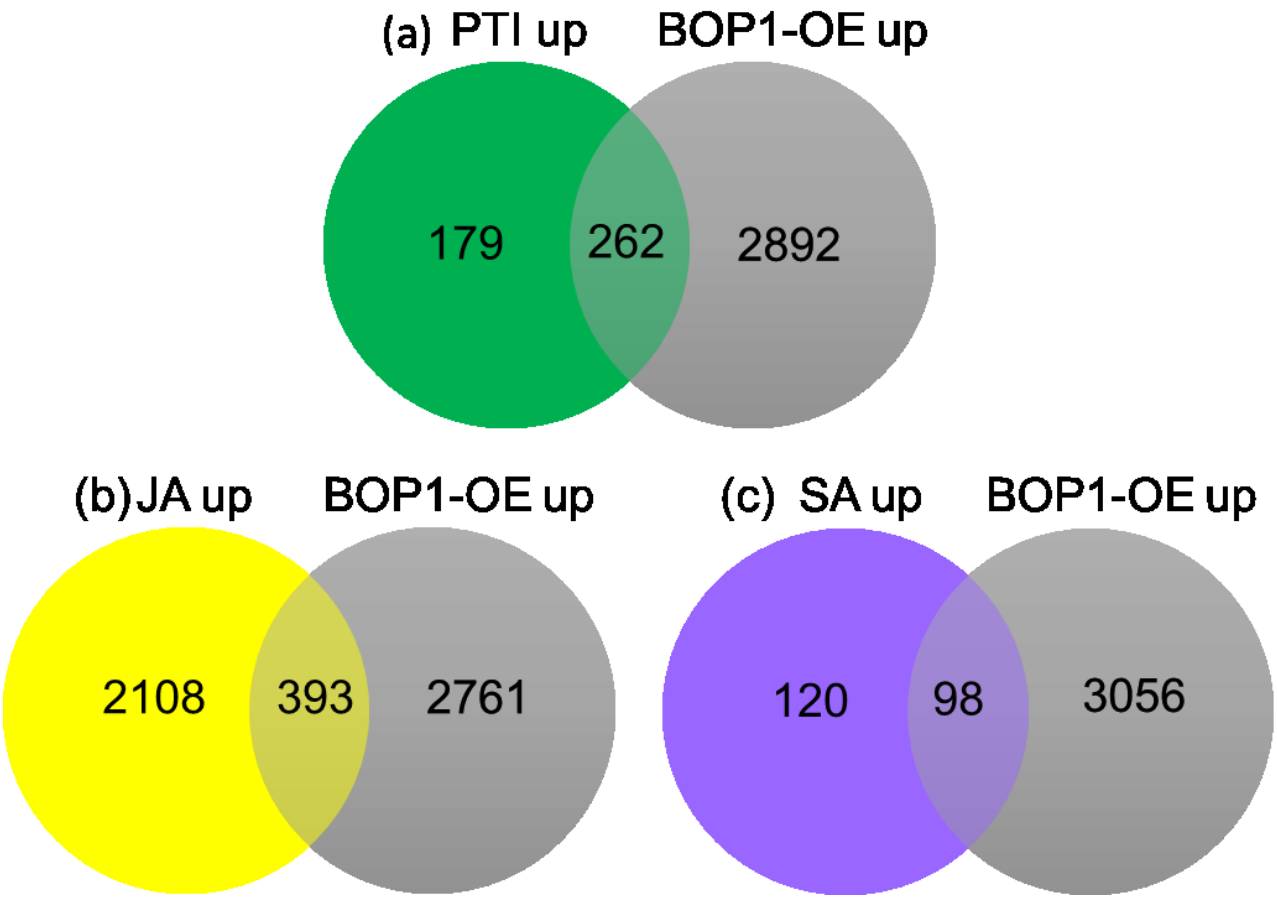
Supplemental Table S1 List of p-values for Fig 3.2

This table lists the p-values obtained for the experimental data shown in Fig 3.2. The p-values were obtained through a one-way ANOVA, followed by a Tukey's post hoc. The significance value was set at p= 0.05, where values lower correspond to an asterisk and represent statistically significant differences. Values higher are denoted as triangles in the figure and are not significant

Genotype	Experimental p-value				
	SARD1	*CBP60g*	*FLS2*	*RBOHD*	*PR2*
WT	0.0480	0.0568	0.0522	0.0158	0.0197
BOP1-OE	0.0005	0.0433	0.0165	0.0091	0.0001
bop1 bop2	0.9969	0.9932	0.9991	1.0000	0.0830
tga1 tga4	0.7367	1.0000	0.9322	0.9992	0.0830
bop1 bop2 tga1 tga 4	1.0000	1.0000	1.0000	0.9410	0.8399
null	0.9827	0.9999	1.0000	0.9997	1.0000
fls2	1.0000	1.0000	1.0000	1.0000	0.6642

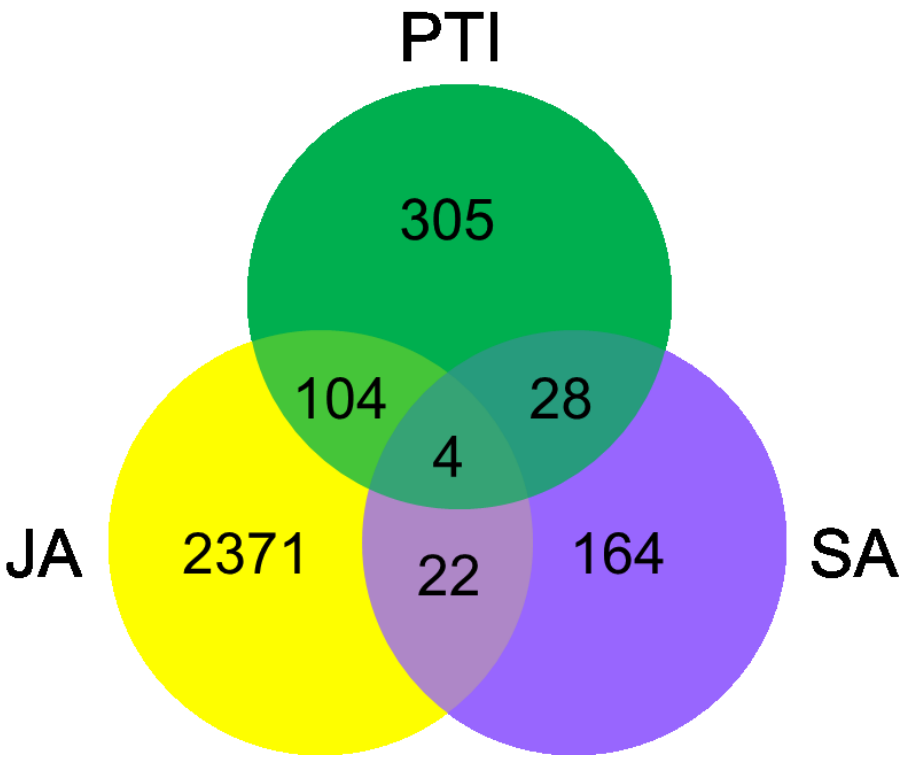
Supplemental Table S2 List of p-values for Fig 3.3

This table lists the p-values obtained for the experimental data shown in Figure 3.3. The p-values were obtained through a one-way ANOVA, followed by a Tukey's post hoc. The significance value was set at p= 0.05, where values lower correspond to an asterisk and represent statistically significant differences. Statistical analysis shows significantly different inductions between treatments of a PAMP for 0 hours and 4 hours.



Supplemental Figure S1. Venn diagrams showing the overlap of genes upregulated in each defense response compared to BOP1-OE microarray.

(a) PTI dataset (Wan *et al.*, 2008) versus BOP1-OE microarray dataset showing an overlap of 262 genes (59%). (b) Dataset for *B. cinerea* which strongly elicits a JA defense response (Windram *et al.*, 2012) versus BOP1-OE microarray dataset showing an overlap of 393 genes (16%). (c) SA dataset (Blanco *et al.*, 2009) versus BOP1-OE microarray dataset showing an overlap of 98 genes (44%). Data were sorted using GeneVenn's online tool (Pirooznia *et al.*, 2007).



Supplemental Figure S2. Three-way Venn diagram showing the overlap of upregulated genes between each defense response.

This diagram compares each of the defense responses to one another to depict overlap between datasets. The data were sorted using GeneVenn's online tool (Pirooznia *et al.*, 2007).