**Investigating the roles of BLADE-ON-PETIOLE1 and 2 with clade I TGACG-motif
binding basic leucine zipper transcription factors in the regulation of development and
defense in *Arabidopsis thaliana***

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ABSTRACT

Plant health relies on a critical balance between growth and defense. The activation of defense responses is generally linked to growth repression but the mechanisms behind this response are unclear. BTB-ankyrin domain-containing proteins comprise a family of transcriptional co-regulators in land plants with roles in development and defense. Discovered in *Arabidopsis thaliana*, proteins in this family form two groups: NONEXPRESSOR OF PATHOGENESIS RELATED GENES 1 (NPR1) and related proteins are essential for systemic acquired resistance to pathogens whereas BLADE-ON-PETIOLE 1 and 2 proteins regulate plant morphology. Structurally, proteins from both groups lack a DNA-binding domain but share conserved protein-interacting BTB/POZ and ankyrin domains. BTB-ankyrin proteins interact with TGACG-motif binding (TGA) basic leucine zipper (bZIP) and WRKY transcription factors but functional knowledge of these interactions is incomplete.

My thesis explores roles for BOP1 and BOP2 in development and defense with TGA bZIP transcription factors as functional partners. First, I showed that clade I TGAs function in the same genetic pathways as BOP1/2 important for the development of organ boundaries and plant architecture. Second, I showed that BOP1/2 function in pattern-triggered immunity through interaction with clade I TGA and WRKY transcription factors. Lastly, I found that BOP1/2 regulate plant defense in an NPR1-independent manner. Collectively, these findings revealed a dual role of BOP1/2 in plant development and defense.

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PREFACE

This dissertation identifies previously undiscovered partners of BLADE-ON-PETIOLE1 (BOP1) and BOP2 in regulating plant architecture and immune responses. This work has resulted in one first-author publication and one submitted manuscript. I contributed the majority of results included in this thesis. However, the data were obtained as a result of collaboration with others. The detailed contributions are listed below.

Statement of Contributions

1. Chapter 2

Published in Plant Physiology.

Wang Y., Salasini B.C., Khan M., Devi B., Bush M., Subramaniam R., Hepworth S.R. (2019) Clade I TGACG-motif binding basic leucine zipper transcription factors mediate BLADE-ON-PETIOLE-dependent regulation of development. *Plant Physiol.* **180**: 937-951

Ying Wang: analyzed *tgal pny pnf*, *tga4 pny pnf*, and *tgal tga4 pny pnf* mutants; profiled the expression pattern of *TGAL* and *TGA4* in *pny*, *pnf*, and *pny pnf* backgrounds; tested the *ATH1* expression pattern using reporter genes and RT-qPCR; created the homozygous *ATH1p:GUS BOP1-GR tgal tga4* line; generated *pTGAL:TGAL-GFP tgal tga4* and *pTGA4:TGA4-GFP tgal tga4* mutants; performed chromatin immunoprecipitation assays, performed bimolecular fluorescence complementation assays, performed yeast two-hybrid assays; drafted the manuscript.

Brenda C. Salasini: characterized *tgal pny pnf*, *tga4 pny pnf*, and *tgal tga4 pny pnf* mutants.

Madiha Khan: initiated the project and characterized the initial crosses of *tgal*, *tga4*, and *tgal tga4* with *35S:BOP2*.

Bhaswati Devi: initial characterization of *TGA1* and *TGA4* expression pattern using *TGA1p:GUS* and *TGA4p:GUS* reporter genes.

Michael Bush: generated the *TGA1p:GUS* and *TGA4p:GUS* reporter genes.

Rajagopal Subramaniam: analyzed protein-protein interactions in conjunction with Y.W. and provided access to confocal microscopy.

Shelley R. Hepworth: designed the experiments, supervised the project, performed the initial crosses of *tgal*, *tga4*, and *tgal tga4* mutants with *pnf*, *pnf*, and *pnf pnf* mutants; prepared the manuscript in conjunction with Y.W.

2. Chapter 3

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Ying Wang carried out Gene Ontology (GO) and Gene Set Enrichment Analysis (GSEA) of *BOP1-oe* microarray data in collaboration with C.J.B.; analyzed hydrogen peroxide and callose production in wild-type and mutant seedlings under control conditions, analyzed RNA-sequencing results and performed the bioinformatics analysis; performed chromatin immunoprecipitation

assays, performed yeast-two-hybrid assays, collected and prepared samples for measurement of salicylic acid; drafted the manuscript.

Chris Bergin: performed GO and GSEA analysis of *BOP1-oe* microarray data in conjunction with Y.W.; performed RT-qPCR experiments to validate the microarray and demonstrate co-regulation of select PTI-related genes by BOP1/2 and TGA1/4; and performed growth inhibition assays.

Bodunde Oyetoran: tested susceptibility of wild-type and mutants to *Pseudomonas syringae* and *Botrytis cinerea* pathogens, quantitatively measured oxidative burst and callose deposition in wild-type and mutants.

Steven Chatfield: isolated RNA and analyzed raw *BOP1-oe* microarray data.

Raju Datla: provided raw microarray data for *BOP1-oe* plants with D.X.

Daoquan Xiang: provided raw microarray data for *BOP1-oe* plants with R.D.

Yuanchu Liu: isolated total RNAs of *bop1 bop2*, *tgal tga4*, *BOP1-oe*, and wild-type seedlings after flg22-treatment with Y.W. and L.L.

Lin Li: tended *bop1 bop2*, *tgal tga4*, *BOP1-oe*, and wild-type seedlings for flg22-treatment, and participated in total RNA isolation with Y.L. and Y.W.

Zhezhi Wang: supervised Y.L. and L.L.; provided experimental space, reagents, and instruments for RNA-sequencing.

Jenna O'Neill: performed GO analysis of RNA sequencing data for construction of figures and tables in collaboration with Y.W. and S.R.H.

Chris Bonner: analyzed salicylic acid (SA) samples by HPLC in conjunction with N.M.

Nimrat Manes: analyzed SA samples with C.B.

Myron Smith: supervised B.O. and performed statistics analysis on bacterial susceptibility assays.

Rajagopal Subramaniam: supervised C.B. and N.M.; provided instruments for oxidative burst and SA measurements.

Shelley R. Hepworth: supervised the work, designed the experiments; analyzed the data and prepared the manuscript with Y.W.

3. Chapter 4

Ying Wang: designed and performed the experiments, analyzed the data,

Dr. Shelley R. Hepworth: supervised the work, assisted with data analysis.

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LIST OF ABBREVIATIONS

3-AT	3-amino-1,2,4-triazole
ABA	abscisic acid
ANK	ANKYRIN
<i>as-1</i>	<i>activation sequence-1</i>
<i>ATH1</i>	<i>ARABIDOPSIS THALIANA HOMEBOX GENE1</i>
BAK1	BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE1
BELL	BEL1-like
BiFC	Bimolecular fluorescence complementation
<i>BIK1</i>	<i>BOTRYTIS-INDUCED KINASE1</i>
<i>BOP1/2</i>	<i>BLADE-ON-PETIOLE1</i> and 2
BTB	Broad Complex, Tramtrack, and Bric-à-Brac
bZIP	basic region, leucine zipper motif
<i>CaMV35S/35S</i>	Cauliflower Mosaic Virus 35S RNA promoter
<i>CBP60g</i>	<i>CALMODULIN BINDING PROTEIN 60-LIKE</i>
<i>CERK1</i>	<i>CHITIN ELICITOR RECEPTOR KINASE1</i>
ChIP	Chromatin Immunoprecipitation
CZ	central zone
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DDO	double drop-out
DEGs	differentially expressed genes

DEX	dexamethasone
ET	ethylene
ETI	effector-triggered immunity
<i>FLS2</i>	<i>FLAGELLIN SENSITIVE2</i>
GA	gibberellic acids
GO	Gene Ontology
<i>GR</i>	Glucocorticoid receptor
GSEA	Gene Set Enrichment Analysis
His	histidine
HR	hypersensitive reaction
IM	inflorescence meristem
ISR	induced systemic resistance
JA	jasmonic acid
<i>KNAT2/6</i>	<i>KNOTTED-LIKE FROM ARABIDOPSIS THALIANA2</i> and <i>6</i>
KNOX	KNOTTED1-like
LB	Lysogeny Broth
Leu	leucine
LOB	Lateral organ boundary
MS	Murashige and Skoog
<i>NPRI/2/3/4</i>	<i>NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1</i> , <i>2</i> , <i>3</i> , and <i>4</i>
nt	nucleotide
PAMPs	pathogen-associated molecular patterns
PAN	PERIANTHIA

PCA	Principle Component Analysis
<i>PDF1.2</i>	<i>PLANT DEFENSIN1.2</i>
<i>PNF</i>	<i>POUND-FOOLISH</i>
<i>PNY</i>	<i>PENNYWISE</i>
POZ	POX virus and Zinc finger
<i>PR</i>	<i>PATHOGENESIS-RELATED</i>
<i>Pst</i>	<i>Pseudomonas syringae</i> pathovar (pv) <i>tomato</i>
<i>Pst HrcC</i>	mutant of <i>Pst</i> lacking a functional type 3 secretion system
PTI	PAMPs-triggered immunity
PZ	peripheral zone
R protein	resistance proteins
<i>RBOHD</i>	<i>RESPIRATORY BURST OXIDASE HOMOLOG PROTEIN D</i>
ROM	root apical meristem
ROS	reactive oxygen species
ROXYs	CC-type glutaredoxins
RT-qPCR	reverse transcription quantitative PCR
RZ	rib zone
SA	salicylic acid
SAM	shoot apical meristem
SAR	systemic acquired resistance
<i>SARD1</i>	<i>SAR-DEFICIENT1</i>
SD	synthetic defined
TALE	THREE-AMINO ACID LOOP EXTENSION

TDO	triple drop-out
<i>TGA1/4</i>	<i>TGACG-MOTIF BINDING PROTEIN 1</i> and <i>4</i>
TM	tunicamycin
Trp	tryptophan
WT	wild-type
YFP/GFP	yellow/green fluorescent protein

GENETIC NOMENCLATURE CONVENTIONS

<i>BOP1</i>	wild-type gene
<i>bop1</i>	loss-of-function mutant
<i>BOP1-oe</i>	<i>BOP1</i> overexpression (gain-of-function) mutant
BOP1	wild-type protein
<i>BOP1p</i>	<i>BOP1</i> promoter
BOP1-GFP	translational fusion of BOP1 to GFP protein
<i>BOP1p::GFP</i>	transcriptional fusion of <i>BOP1</i> promoter to <i>GFP</i> gene

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CHAPTER 1

INTRODUCTION

1.1 Thesis overview

Plant fitness requires a complex balance between growth and defense. In most plants, pathogen attack results in a suspension of growth as resources are diverted towards defense. Understanding the mechanisms behind this trade-off can benefit the breeding of crops that have better fitness.

In this thesis, the model plant species *Arabidopsis thaliana* (Arabidopsis) was used to explore the role of BLADE-ON-PETIOLE (BOP) transcription co-factors in regulating development and defense. These and other members of the BTB-ankyrin family are recognized by two conserved protein-protein interaction motifs: a BTB/POZ (Broad Complex, Tramtrack, and Bric-à-Brac/POX virus and Zinc finger) domain located in the N-terminus and an ankyrin repeat domain near the C-terminus (reviewed in Khan et al., 2014). As transcription co-factors, these proteins lack a DNA binding domain and form complexes with other transcription factors to regulate gene expression (Backer et al., 2019; Khan et al., 2014).

Arabidopsis BTB-ankyrin proteins are a six-member family. These proteins fall into two phylogenetic subgroups: a primary subclade comprising NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) and its three close homologs (NPR2, NPR3, and NPR4) with roles in plant defense and a secondary subclade comprising BOP1 and BOP2 with roles in plant development (Khan et al., 2014). NPR1 and its closest relatives are master defense regulators of systemic acquired resistance (SAR) (reviewed in Backer et al., 2019) whereas BOP1 and BOP2 regulate plant architecture, mainly through their activity at organ boundaries (reviewed in Pautot and Hepworth, 2015). Boundaries are specialized regions found at the base of organs at joints with the plant body. In *bop1 bop2* mutants, expanded growth at boundaries causes fused

organs, an increased complexity of leaves and flowers, branching defects, and loss of abscission (Norberg et al., 2005; Hepworth et al., 2005). Contrariwise, plants that overexpress *BOP1* (*BOP1-oe*) or *BOP2* (*BOP2-oe*) are repressed in growth resulting in meristem, leaf, and inflorescence phenotypes (Hepworth and Pautot, 2015).

BOP1/2 have long been speculated to have a role in plant defense based on their similarity to *NPR1*, but evidence was lacking. Two classes of transcription factors serve as functional partners for *NPR1* in plant defense: various TGA (TGACG-motif binding) bZIP and WRKY transcription factors (Zhang et al., 1999; Zhou et al., 2000; Despres et al., 2000; Després et al., 2003; Boyle et al., 2009). While *BOP1* and *BOP2* also require TGA partners for developmental activity, no such role for WRKY transcription factors has been described. My thesis finds evidence of a dual role for *BOP1/2* in development and defense using TGA and WRKY functional partners. These data broaden our knowledge of the plant immune system.

1.2 *Arabidopsis thaliana* as a model plant

Arabidopsis thaliana (*Arabidopsis*, also known as mouse ear or thale cress) is a small flowering plant belonging to the Brassicaceae family. This plant is a close relative of commercially important mustard crops including cabbage, canola, and radish. *Arabidopsis* was first proposed as a model plant in the early 1960s (reviewed in Meinke et al., 1998). Although *Arabidopsis* itself has no agronomic value, many traits of this species make it well-suited for botanical studies. *Arabidopsis* plants are short (roughly 20-25 cm tall) and easy to grow in controlled environments. Under continuous light conditions, a complete life cycle is only six weeks long and each plant generates a relatively high number of seeds. Well-established and simple genetic modification methods for *Arabidopsis* have been in place since the mid 1980s (Feldmann and David Marks, 1987; Zhang et

al., 2006). Because of this ease, multiple gene knockout and gain-of-function mutant pools are available for research through stock centers (i.e., Arabidopsis Biological Resource Center). Arabidopsis has the added distinction of being the first plant with a completely sequenced genome (Kaul et al., 2000). More than 20,000 genes were identified in Arabidopsis genome sequence. Because of the wide adoption of Arabidopsis in the research community, gene annotations have been constantly updated and provide a great tool for large scale genomic and bioinformatics studies. The simple structure of an Arabidopsis plant makes it relatively easy to identify and score morphological defects. The interactions between Arabidopsis and pathogens such as the bacteria *Pseudomonas syringae* (*P. syringae*) or the fungus *Botrytis cinerea* (*B. cinerea*) represent useful model systems in which to explore the mechanism by which plants defend themselves against pathogens (reviewed in Katagiri et al., 2002; Govrin and Levine, 2002). Overall, the vast genetics and genomics knowledge gained from Arabidopsis has been successfully applied to the breeding of economically valuable crops (reviewed in Gepstein and Horwitz, 1995; Li et al., 2017; Borrill, 2020).

1.3 General concepts of Arabidopsis shoot development

The life cycle of an Arabidopsis plant involves three phases of embryonic, vegetative, and reproductive development that are common to flowering plants. Within a developing seed, the fertilized zygote undergoes cell divisions to form an embryo with a basic set of structures: a pair of leaves (cotyledons) and root (radical) connected by a stem (hypocotyl). Two populations of stem cells are formed at opposites poles of the embryo: the shoot apical meristem (SAM) and the root apical meristem (RAM). Germination of the seed begins vegetative development. The SAM develops all of the above-ground structures of a plant including leaves, stems, and flowers. The

RAM develops the root system (Aichinger et al., 2012). My thesis will focus on the regulatory mechanisms governing the Arabidopsis above-ground parts generated from SAM.

Plant shoot development by the SAM involves the production of repeating “building blocks” called phytomers (Schultz and Haughn, 1993). Each phytomer consists of a leaf with an axillary meristem attached at the junction called a node to a subtending segment of the stem called an internode (reviewed in Carles and Fletcher, 2003). During vegetative development, the SAM produces a compact rosette of leaves without stem elongation (Figure 1.1). The transition to reproductive development is controlled by intrinsic genetic programs and external environmental cues (Amasino and Michaels, 2010; Irish, 2010). Upon integration of these signals, the SAM becomes a reproductive inflorescence meristem (IM) that bears flowers. Flowers produced by the IM are the product of rapidly proliferating axillary meristems that develop in the axil of leaves (Figure 1.1). Early during the transition to flowering, axillary meristems formed by the IM develop as branches. Leaf development is partially repressed during this stage so that each branch is subtended by a cauline leaf at the node. Each branch is separated by a long internode. When the transition to flowering is complete, axillary meristems formed by the IM develop as solitary flowers where leaf development is fully repressed resulting in single flowers separated by long internodes on the main stem (Figure 1.2). Each of the specialized organs that make up a flower (sepals, petals, stamens, and carpels) can be considered as a modified leaf (Talbert et al., 1995). As such, the transition from one type of phytomer to another is strictly governed at a genetic level (Schultz and Haughn, 1993). All of the structures produced by the SAM and its derivatives develop in step-wise fashion, one unit at a time, to build the aerial parts of an Arabidopsis plant. This mechanism allows the SAM to produce diverse architectures. Understanding how the SAM is organized and regulated provides important basic knowledge of plant development.

1.3.1 Organization of the SAM

The SAM contains a core of stem cells that divide to maintain themselves and provide daughter cells for organ production. In *Arabidopsis* and other plants, the SAM is a layered structure organized by functional zones (Figure 1.2). When viewed in longitudinal cross-section, the central zone (CZ) at the meristem apex contains a few layers of slowly dividing pluripotent stem cells. Daughter cells made by the CZ contribute to the peripheral zone (PZ) where cell division is more rapid and organs are initiated. The organ-meristem interface is marked by a boundary where growth is restricted and axillary meristems can form. The rib zone (RZ) beneath the CZ contains an organizing center that maintains the CZ and produces cells for upward growth of the stem (Fletcher and Meyerowitz, 2000; Bäurle and Laux, 2003; Barton, 2010; Murray et al., 2012). Differential regulation of activities in the CZ, PZ, boundaries, and RZ controls the changing production of leaves, stems, branches, and flowers throughout the life cycle (Barton, 2010; Aichinger et al., 2012; Bencivenga et al., 2016).

1.3.2 Organ boundaries

Plant architecture is highly dependent on organ boundaries. When viewed in longitudinal cross-sections, the boundary between the meristem and an emerging organ appears as a shallow groove (Aida and Tasaka, 2006). Cells in this region have a slow rate of division and relatively stiff walls that can withstand compression caused by the faster growth of surrounding tissues (Žádníková and Simon, 2014). These important regions of separation between the meristem and a developing organ become a joint where organs connect to the plant body. Boundary cells have the unique potential to form axillary meristems that give rise to lateral branches, flowers, and other

specialized structures like stipules or nectaries. Boundaries also have the potential to form abscission zones where organs can be shed from the plant body (Hepworth and Pautot, 2015).

1.3.3 Molecular mechanism governing SAM maintenance

Maintenance of the SAM relies on two connected mechanisms. A CLAVATA signaling pathway regulates the activity of a WUSCHEL homeodomain transcription factor in a negative feedback loop. This loop renews the CZ and controls its size (Lee and Clark, 2013; Aichinger et al., 2012). Three-amino-acid-loop-extension (TALE) homeodomain transcription factors play a complementary role and act primarily to inhibit stem cell differentiation (Hamant and Pautot, 2010; Aichinger et al., 2012).

The TALE homeodomain superfamily contains KNOTTED1-like (KNOX) and BEL1-like (BELL) subtypes that function as heterodimer pairs (Hamant and Pautot, 2010). The KNOX member SHOOTMERISTEMLESS (STM) plays a central role in maintaining the SAM. Strong *stm* loss-of-function mutants are unable to replenish their stem cell population and initiate only a few leaves (Long et al., 1996; Hamant and Pautot, 2010). Two related BELL homeodomain factors PENNYWISE (PNY) and POUND-FOOLISH (PNF) preserve the stem cell core by maintaining organ boundaries (Smith et al., 2004; Khan et al., 2015). *PNY* expresses broadly in the meristem whereas *PNF* expresses mainly in the CZ and PZ. Both products interact with KNOX partners in the region (Smith and Hake, 2003; Smith et al., 2004; Bencivenga et al., 2016). Loss-of-function *pnf* mutants have defects in the sequence of organ initiation and irregular internode elongation, resulting in clusters of lateral branches and flowers spaced along the inflorescence (Byrne et al., 2003; Smith and Hake, 2003). Simultaneous mutations in both PNY and PNF cause full or partial SAM arrest. Full arrest results in a phenotype similar to the *stm* mutant (Ung and Smith, 2011;

Rutjens et al., 2009). In either case, SAM responsiveness to floral inductive signals is blocked resulting in a non-flowering phenotype (Smith et al., 2004) (Figure 1.2).

Recent studies show that *pnv* and *pnv pnf* defects are due to the misexpression of boundary genes (Figure 1.2). In *pnv* mutants, boundary genes are misexpressed in the RZ and stem cortex causing defects in the elongation of internodes (Ragni et al., 2008; Khan et al., 2012ab). In *pnv pnf* mutants, boundary genes are misexpressed throughout the SAM resulting in meristem arrest or insufficiency (Khan et al., 2015). The inactivation of *BOP1/2* restores meristem function in *pnv* and *pnv pnf* mutants showing an inhibitory effect of boundary genes on stem cell maintenance. To function in boundaries, the BTB-ankyrin proteins BOP1/2 require the downstream activity of KNOX gene *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA6 (KNAT6)* and its BELL functional partner *ARABIDOPSIS THALIANA HOMEODOMAIN GENE1 (ATH1)* (Figure 1.2). The inactivation of these genes also restores meristem function in *pnv* and *pnv pnf* mutants (Ragni et al., 2008; Li et al., 2012; Khan et al., 2015). A genome-wide screen found that PNY directly represses many genes involved in the development of organ boundaries showing this activity to be important for meristem integrity (Bencivenga et al., 2016).

1.4 Growth-defense trade-offs

Plants are constantly exposed to environmental stresses. Unlike animals that can move to a new location, plants are immobile. Each cell can arrest growth and switch to defense. When conditions are favourable, energy is directed towards cell growth and development but when biotic and/or abiotic stresses are encountered, cells shift into defense mode and growth is repressed (Huot et al., 2014).

Growth-defense trade-offs have significant implications in agriculture as both are critical for plant fitness. Although decades of research have investigated the mechanisms governing this switch, the picture is far from clear (Belkhadir et al., 2014; Lozano-Durán and Zipfel, 2015; Jiménez-Góngora et al., 2015). Hormone crosstalk has emerged as a focal point. Various hormone signaling pathways that promote cell division and growth are generally considered as negative regulators of plant defense including auxin, brassinosteroids (BR), and gibberellic acids (GA). Some pathogens, including *P. syringae*, can promote disease in target plants by synthesizing auxin (reviewed in Huot et al., 2014). Coincidentally, boundaries sitting in between the SAM and newly emerged organ primordia are auxin minima (Vernoux et al., 2010). *BOP1/2* are marker genes of boundaries where auxin is excluded and responses to BR and GA are repressed to minimize growth (Vernoux et al., 2010; Hepworth and Pautot, 2015). Mutations in genes encoding major players in BR signaling and biosynthesis result in suppression of growth and infertility (Lozano-Durán and Zipfel, 2015; Wang et al., 2015, 2013). The exact mechanism by which BR integrates with plant defense is still inconclusive, but several lines of evidence depict a negative role of BR in innate immunity. For instance, endogenous and/or exogenous BR treatment suppresses Arabidopsis resistance against bacterial pathogens (Belkhadir et al., 2012; Albrecht et al., 2012). The underlying mechanism has been described as competition between BR and defense signaling pathways for a shared cell-membrane localized receptor BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1) which perceives both BR signaling and bacterial pathogens (Belkhadir et al., 2012; Albrecht et al., 2012). One study identified BOP1 as a negative regulator of BR responses. Genetic study of a brassinazole-sensitive-short hypocotyl (*bss1-ID*) activation-tagged *BOP1* mutant identified BOP1 as a negative regulator of BR signaling. Further biochemical studies including bimolecular fluorescence complementation assays revealed that in the cytoplasm,

BOP1 interacts with the BZR1 transcription factor thereby preventing its translocation into nucleus and lowering BR responses (Shimada et al., 2015). GA is involved in various aspects of plant development including seed germination, promotion of flowering, stem elongation, and organ growth. The GA stimulates the degradation of DELLA repressors to activate downstream signaling responses. Not until recently has the function of GA been tied with defense mechanisms (reviewed in De Bruyne et al., 2014). A higher order mutant of *della* repressors showed elevated levels of SA-dependent resistance against *P. syringae* (Navarro et al., 2008). Endogenous GA in the shoot apex promotes the transition from vegetative to reproductive stage (Galvão et al., 2012). Exogenously applied GA has little or effect on flowering or stem elongation in *BOP1-oe* plants suggesting that the late flowering, dwarf phenotype of this mutant is not simply due to lack of GA biosynthesis (Khan et al., 2015). Altogether, it is tempting to speculate that BOP1/2 repression of growth at boundaries is somehow connected a broader role in defense using NPR1-like mechanisms.

1.5 The immune system of Arabidopsis

Plants use complex strategies to resist pathogens. External barriers such as bark or cuticle cover the outer surface of plants as general forms of protection. Pathogens that overcome these barriers encounter the plant immune system, which includes both innate (local) and induced (systemic) responses (reviewed in Jones and Dangl, 2006). Innate immunity can be divided into pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) or effector-triggered immunity (ETI) which are often deployed sequentially in response to a local infection (Jones and Dangl, 2006; Dangl et al., 2013). Various hallmarks have been identified as representing PTI and ETI responses in Arabidopsis. Generally, a successful PTI response leads to fortification of cell-wall

associated physical barriers and release of chemicals to suppress pathogen growth and colonization. Subsequent ETI is based on cytoplasmic pathogen receptors that initiate programmed cell death called hypersensitive response (HR) (Pieterse et al., 2012; Dangl et al., 2013). If the infection is sufficient, plants can further mount a systemic acquired resistance (SAR) response to protect distal parts of the plant against pathogen invasion (Mou et al., 2003). Alternatively, if the detected microbes are not invasive, plants may activate another response named induced systemic resistance (ISR) which can further boost plant defenses to give elevated resistance (Van Der Ent et al., 2009; Van Loon et al., 1998). Although the outcomes of different immune responses are distinctive from each other, they do share some common regulatory networks. Phytohormones play essential roles in the mediation of various defense branches. The major hormones associated with plant immunity are salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Bigeard et al., 2015). All three hormones are induced at the site of attack, but they serve differently during the various immune responses. It is well known that these hormone-regulated pathways are interconnected, but often, antagonism is observed between them (Kunkel and Brooks, 2002; Denoux et al., 2008; Tsuda et al., 2009; Caarls et al., 2015). In general, SA signaling is the dominant response to biotrophic pathogens (require a living host to complete their life cycle) whereas jasmonic acid (JA)/ethylene (ET) signaling is the dominant response to necrotrophic pathogens (do not require a living host to complete their life cycle) and chewing insects (Glazebrook, 2005; Pieterse et al., 2012).

1.5.1 Innate immunity

Herbaceous plants are naturally protected from pathogen infection by a waxy cuticle on their outer surfaces. Pathogens and chewing insects that penetrate this barrier can trigger immunity. The first and most ancient line of defense known as innate immunity (sometimes called basal resistance)

relies on the recognition of foreign objects by specialized receptors. Depending on the locations of these receptors, innate immunity can be further divided into PTI and ETI. When the membrane-localized receptors detect a PAMP, a localized “oxidative burst” is first initiated, resulting in the fortification of cell wall structural components and the production of chemical barriers (e.g. thickening of the cuticle, deposition of callose, lignification of cell walls, and synthesis of antimicrobial compounds) to repel microbe colonization (Jones and Dangl, 2006; Dangl et al., 2013). One of the most well-documented PTI response in *Arabidopsis* is initiated by the perception of a conserved 22-amino-acid fragment of bacterial flagellin named flg22 (Figure 1.3). Flg22 can be recognized by the membrane localized receptor FLAGELLIN SENSITIVE2 (FLS2) (Gómez-Gómez et al., 1999; Macho and Zipfel, 2014; Gómez-Gómez and Boller, 2000). Under resting condition, FLS2 forms a complex with another membrane kinase BAK1. Upon the perception of flg22, a cytoplasmic kinase, BOTRYTIS-INDUCED KINASE1 (BIK1), is rapidly phosphorylated by BAK1 and released from the FLS2 receptor complex. Phosphorylated BIK1 initiates a signal passing through mitogen activated protein kinase (MAPK) cascades. Meanwhile, a rapid and transient accumulation of reactive oxygen species (ROS) at the attack site is produced by the NADPH oxidase RBOHD at the plasma membrane (Luna et al., 2011; Morales et al., 2016). Both events ultimately lead to the expression of genes involved in the biosynthesis of antimicrobial compounds and the fortification of plant cell walls (Bigeard et al., 2015). Another example of a PAMP is fungal chitin, which can be recognized by the receptor CHITIN ELICITOR RECEPTOR KINASE1 (CERK1, also known as LYSINE MOTIF-CONTAINING RECEPTOR-LIKE KINASE1) (Gómez-Gómez and Boller, 2000; Zipfel et al., 2004).

Some pathogens have evolved mechanisms to bypass PTI by delivering molecules into plant cells called “effectors” that suppress the PTI machinery. One of the best-studied means of

effector delivery is the bacterial type III secretion system of *P. syringae* (Hauck et al., 2003). A *P. syringae hrcC*⁻ mutant lacking the HrcC protein, which is an essential component in assembling the type III secretion system, can be used to directly test Arabidopsis PTI responses. Plant recognition of pathogen effectors by cytoplasmic resistance (R) proteins leads to a stronger race-specific immune response called ETI. A hallmark of this response is a programmed cell death called “hypersensitive response” at the site of infection. Small lesions of cell death at the attack site can prevent the pathogen from further spreading. Both PTI and ETI prevent pathogens from advancing and serve as a priming mechanism to arm the plant against future attacks (Henry et al., 2013). In particular, ETI leads to a localized production of mobile signals that trigger systemic resistance in distant parts of a plant (Pieterse et al., 2012; Dangl et al., 2013). Although the outcomes of PTI and ETI are different, they do share some common signaling pathways (Tsuda et al., 2009), such as the involvement of both SA and JA-dependent signaling pathways, which are activated at the site of attack, but in a localized manner (Betsuyaku et al., 2018).

1.5.2 Systemic acquired resistance

Upon detection of certain biotrophic pathogens, Arabidopsis can extend and enhance localized resistance to its distal parts to ward off infection. This type of induced immune response is known as systemic acquired resistance (SAR). Both localized PTI and ETI responses have the ability to trigger a long-lasting signal leading to SAR (Spoel and Dong, 2012). Genetics studies on Arabidopsis mutants lacking the biosynthesis and translocation of salicylic acid (SA) suggested the central role of this phytohormone in SAR. Many *P. syringae* secrete the phytotoxin coronatine which functions as a mimic of JA-Ile (an amino acid conjugated form of JA). Activation of the JA pathway, which is generally antagonistic to SA signaling, results in a suppression of SA signaling,

as a benefit to the pathogen (Kunkel and Brooks, 2002). In Arabidopsis, SA is synthesized through two major branches including phenylalanine ammonia-lyase and isochorismate synthase-dependent pathways. SA biosynthesis takes place in chloroplasts and requires further transportation into the cytoplasm to function. ENHANCED DISEASE SUSCEPTIBILITY5 (also known as SA INDUCTION-DEFICIENT1) is a toxin-extrusion like transporter localized on the chloroplast envelope that mediates SA translocation into the cytoplasm (Nawrath et al., 2002; Serrano et al., 2013). Mutants lacking these enzymes showed increased susceptibility to pathogen and are unable to mount a successful SAR (Yalpani et al., 1993; Huang et al., 2010). Translocation of SA into the cytoplasm activates a master regulator in plant defense, the NPR1 protein, which in turn activates downstream defense-related genes, including *PATHOGENESIS-RELATED (PR)* marker genes.

Since the discovery of SAR in the 1960s, many studies have suggested that in order to activate SAR, a mobile signal generated at the site of attack must travel through phloem to reach uninfected distal tissues (reviewed in Gao et al., 2015; Tian and Zhang, 2019). Many speculations on SA as the mobile agent in SAR were denied, as a grafting assay on *Nicotiana tabacum* using the root of a mutant with abolished SA biosynthesis can still trigger SAR in the scion, indicating that SA was unlikely to be the mobile signal during SAR. Meanwhile, several other signaling components have been proposed to be the potential mobile agents in SAR, including azelaic acid, pipecolic acid, and their derivatives (Sun et al., 2015). The biosynthesis of these components is complicated and two plant-specific transcription factor genes *SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1)* and *CALMODULIN-BINDING PROTEIN 60g (CBP60g)* have been found to be important for SA biosynthesis during a defense response, and recently further identified as regulators in pipecolic acid biosynthesis. Loss-of-function *sard1 cbp60g*

mutations block both local and systemic accumulation of the SA biosynthesis gene *ISOCHORISMATE SYNTHASE1* resulting in compromised innate immunity and inability to mount SAR (Sun et al., 2018, 2015).

1.5.3 Induced systemic resistance

SAR is not the universal response of *Arabidopsis* against pathogen attacks. Many beneficial bacteria in the rhizosphere and certain necrotrophic pathogens can trigger a different form of induced immunity known as induced systemic resistance (ISR) (van Wees et al., 1999; Mathys et al., 2012). Phenotypically, ISR resembles SAR, in that a localized immune response triggers enhanced resistance in the distal parts of a plant, but the underlying mechanism is different (Verhagen et al., 2004). Due to the similarity between ISR and SAR, the defining criteria of ISR have been constantly amended. In general, ISR is regulated by JA/ET signaling pathways. The plant defense arsenal in an ISR response remains dormant and is only activated under a pathogen attack (Van Hulten et al., 2006). Several marker genes in SAR, such as *PR1* and *PR2*, are not induced in an ISR. Although NPR1 is involved in the ISR response, this role is not essential as in SAR (Backer et al., 2019; Van Wees et al., 2000). Overall, SAR and ISR are governed by different phytohormones, but as research progresses, a great portion of these defense responses might turn out to be overlapping (Withers and Dong, 2016; Pieterse et al., 1998; Mathys et al., 2012; van Wees et al., 1999).

1.6 *Arabidopsis* BTB-ankyrin proteins and their functioning mode

NPR1 is the founding member of a land-plant family of transcriptional co-factors identified by a BTB/POZ domain and ankyrin repeats. The BTB/POZ domain, first characterized in *Drosophila*

developmental regulators including Broad-complex, Tramtrack, and Bric-à-Brac, is an essential domain for homo- or hetero-dimerization (Godt et al., 1993). Ankyrin domains are widespread in nature and mediate protein-protein interactions spanning a wide range of functions (Mosavi et al., 2004). BTB-ankyrin proteins in mosses and higher plants comprise two phylogenetic subclades (Saleh et al., 2011). In *Arabidopsis*, NPR1 and its three close homologs (NPR2, NPR3, and NPR4) form the first subclade with roles in plant defense (Cao et al., 1997; Canet et al., 2010; Fu et al., 2012; Ding et al., 2018; Backer et al., 2019). A second subclade containing BOP1 and BOP2 regulate plant development (Ha et al., 2003; Chan et al., 2004; Hepworth et al., 2005; Norberg et al., 2005). Structurally, all six BTB-ankyrin proteins share the BTB/POZ domain and ankyrin repeats, but NPR1-like proteins have a divergent C-terminus compared to BOP1 and BOP2 (Figure 1.4). The C-terminus of NPR1 contains a bipartite basic nuclear localization signal and a transactivation domain (Rochon et al., 2006; Wu et al., 2012b). All six BTB-ankyrin proteins have irreplaceable roles in *Arabidopsis*. To date, two major functioning modes for both subclades have been identified. First, these proteins function as transcription co-activators (NPR1/BOP1/BOP2) or co-repressors (NPR3/NPR4) in regulating target gene expression (Després et al., 2003; Jun et al., 2010; Ding et al., 2018). Proteins from both subclades lack a known DNA-binding domain and interact with TGA bZIP transcription factors for this purpose (Zhang et al., 1999; Zhou et al., 2000; Despres et al., 2000; Després et al., 2003; Boyle et al., 2009). Second, proteins from both subclades can regulate protein degradation by serving as an adaptor to Cullin 3 ubiquitin E3 ligases (Fu et al., 2012; Chahtane et al., 2018; Zhang et al., 2017).

1.6.1 NPR1 and NPR1-like proteins

A role for NPR1 in plant defense was first identified based on loss-of-function mutations that eliminated SAR (Cao Hui et al., 1994; Cao et al., 1997). Following local infection, elevated levels of SA in the cytoplasm lead to monomerization of NPR1, allowing its transportation into the nucleus (Mou et al., 2003; Tada et al., 2008). In the nucleus, NPR1 interacts with various TGA bZIP transcription factors, thereby activating the expression of defense genes such as *PR1*. NPR1 contains a novel Cys-containing transactivation domain that is sensitive to SA. C521/C529 residues in this C-terminal domain of NPR1 interact with SA via copper ions. SA binding triggers a conformational change in NPR1 that promotes its activity allowing interaction with TGA2, TGA5, and TGA6 factors and activation of defense genes (Rochon et al., 2006; Wu et al., 2012a).

All proteins in the NPR1 subclade can perceive SA, but only NPR2 can partially complement the defense defects of the loss-of-function *npr1* mutant (Castelló et al., 2018). Different from NPR1 and NPR2, NPR3 and NPR4 function negatively in plant defense (Fu et al., 2012). Characterization of loss-of-function *npr3 npr4* mutant showed a higher level of NPR1 proteins accompanied with decreased induction of SAR after challenging plant with pathogens. Studies found that NPR3 and NPR4 can physically bind with NPR1 and serve as adaptors to Cullin 3 ubiquitin E3 ligases in mediating NPR1 degradation (Fu et al., 2012). As well, NPR3 and NPR4 act as co-repressors proving that both proteins are *bona fide* SA receptors and transcriptional regulators (Ding et al., 2018). The transcript levels of *SARD1* and *WRKY70* were elevated in a *npr3 npr4* double mutant, and this induction was not attenuated in an *npr1 npr3 npr4* triple mutant indicating that NPR3 and NPR4 function independently from NPR1 (Fu et al., 2012).

1.6.2 BOP1/2 regulate plant development and defense

Arabidopsis BOP1 and BOP2 proteins form a distinct subclade within the NPR1 family. Structurally, BOP1 and BOP2 share the conserved BTB/POZ and ankyrin repeat domains. In aerial tissues, *BOP1* and *BOP2* expression is enriched at organ boundaries and control development in this region (Richardson and Hake, 2019; Khan et al., 2014). Single mutants in Arabidopsis have little or no phenotype suggesting that BOP1 and BOP2 function redundantly (Hepworth et al., 2005; Khan et al., 2014). Major boundary-related defects are observed in *bop1 bop2* double mutants including organ fusions, elongated leafy petioles, abnormal floral patterning, and loss of abscission (Norberg et al., 2005; Hepworth et al., 2005). BOP1/2 also regulate lignin deposition during secondary growth in root, stem, and hypocotyl (Khan et al., 2014; Liebsch et al., 2014; Woerlen et al., 2017).

Recent studies suggest that BOP1/2 might have a defense-related role. Defects in methyl jasmonate induced defense against *P. syringae* were reported in Arabidopsis *bop1 bop2* mutants (Canet et al., 2012). In cotton plants, *Gossypium hirsutum* GhBOP1 can be spatially induced outside of organ boundaries in the stem to defend against fungal pathogen *Verticillium dahliae* through interaction with GhTGA3 and the deposition of lignin (Zhang et al., 2019). The homologs of BOP1/2 in legume also control symbiosis with rhizobia (Couzigou et al., 2012; Magne et al., 2018).

TGACG-motif binding (TGA) basic leucine zipper (bZIP) transcription factors can recruit BTB-ankyrin proteins to DNA (Backer et al., 2019). In flowers, BOP1/2 interact with TGA8 (also known as PERIANTHIA/PAN). A distinctive fifth organ is formed in the sepal and petal whorls on the abaxial side of *bop1 bop2* and *pan* flowers showing a shared role in floral patterning

(Hepworth et al., 2005; Xu et al., 2010). We have long hypothesized that BOP1/2 activity outside of flowers relies on other TGA factors but evidence has been lacking. BOP1/2 also serve as substrate adaptors of CULLIN3 ubiquitin ligase complex to regulate LEAFY and PHYTOCHROME INTERACTING FACTOR 4 post-translationally to regulate plant development (Zhang et al., 2017; Chahtane et al., 2018). These isolated studies point to a dual role for BOP1 and BOP2 in development and defense using mechanisms similar to proteins of the NPR1 subclade.

1.7 The role of TGA transcription factors in plant development and defense

The transcription of a gene is controlled by small *cis* elements mainly present in non-coding genomic DNA preceding the coding region, known as a promoter. Differences in the variety, quantity, as well as distribution of different *cis* elements governs the strength and inducibility of a promoter. Early studies of *cis*-elements in plants were driven by investigation of plant-microbe interactions. In dicot plants, one of the best characterized and useful promoters is the Cauliflower Mosaic Virus 35S RNA promoter (*CaMV35S*, 35S) (Benfrey and Chua, 2011). Within the 35S promoter, there are two TGACG motifs in tandem found at nucleotides -65 to -85 relative to the transcriptional start site (+1) known as the *activation sequence-1* (*as-1*) region (Lam et al., 1989). This *as-1* sequence was first identified to bind with tobacco TGACG-motif binding (TGA) 2.1 and TGA2.2 which belong to the bZIP transcription factor super family. TGA transcription factors were once named as OCTOPINE SYNTHASE-ELEMENT BINDING FACTOR because the *as-1* sequence was also found in the promoter of octopine synthase coding genes from *Agrobacterium tumefaciens* which causes crown gall tumors (reviewed in Gatz, 2013). The *as-1* element as well

as the later discovered *as-1*-like sequences have been found in the promoters of multiple SA-, auxin-, methyl jasmonate-responsive genes.

In Arabidopsis, there are ten TGA transcription factors, which share a conserved domain "Yx2RL[RQ]ALSS[LS]W" that distinguishes them from other members of the bZIP transcription factor superfamily (Schindler et al., 1992; Izawa et al., 1993; Jakoby et al., 2002). Based on phylogenetic analysis, these ten TGAs can be divided into 5 subclades: clade I comprises TGA1 and TGA4; Clade II comprises TGA2, 5, and 6; clade III comprises TGA3 and TGA7; class IV comprises TGA9 and TGA10; and clade V comprises TGA8 (also known as PERIANTHIA/PAN) (Gatz, 2013; Jakoby et al., 2002; Zander et al., 2010; Shearer et al., 2012; Noshi et al., 2016; Zhang et al., 1999). The functions of different members of the TGA subfamily varies in different contexts. Briefly, clade II and III TGAs have been shown to be involved in plant defense (Zhang et al., 1999; Zander et al., 2010; Després et al., 2003; Boyle et al., 2009); clade V TGA functions solely in plant development (Hepworth et al., 2005; Li et al., 2009; Gutsche and Zachgo, 2016); clade I and IV TGAs have a dual role in both plant defense and development (Wang and Fobert, 2013; Shearer et al., 2012; Noshi et al., 2016; Wang et al., 2019; Sun et al., 2018; Song et al., 2008; Alvarez et al., 2014).

NPR1 interacts with both clade I and II TGA transcription factors to regulate plant defense genes (Despres et al., 2000; Després et al., 2003; Fode et al., 2008; Zhang et al., 1999; Boyle et al., 2009). Clade II TGAs are major players in SA-mediated plant defense (Zander et al., 2012; Caarls et al., 2015). Like *npr1*, a *tga2 tga5 tga6* triple mutant is compromised in SAR and does not express *PR1* upon treatment with SA or a synthetic SA analog 2,6- dichloroisonicotinic acid (Zhang et al., 2003). Biochemical evidence revealed that clade II TGA2, TGA5, and TGA6 proteins can

physically interact with NPR1 to form a complex. In addition, clade II TGA factors are also involved in defense pathways regulated by JA/ET (Zander et al., 2010, 2012). Transcriptional activation of PLANT DEFENSIN1.2 (*PDF1.2*), a marker gene of the JA/ET response, depends on clade II TGA factors after application of the ET precursor 1-aminocyclopropane-carboxylic acid, either alone or in combination with JA, and after *B. cinerea* infection (Zander et al., 2010). This is very interesting, as the same group of TGA transcription factors are not only essential for SA-mediated transcription genes such as *PR1*, but also critical for ET-mediated induction of *PDF1.2*, but JA can bypass clade II TGA transcription factors and activate *PDF1.2* (Zander et al., 2010, 2014). Generally, SA-mediated and JA/ET-mediated defense pathways have an antagonistic effect on each other. A requirement in SA-mediated repression of *PDF1.2* by clade II TGAs implies their potential activity as switch between SA and JA/ET defense pathways (reviewed in Gatz, 2013).

Clade I TGA1 and TGA4 contain intramolecular disulphide bonds under normal cellular conditions that prevent constitutive interaction with NPR1. However, reduction of these residues following SA treatment enables an interaction with NPR1 (Després et al., 2003). Nevertheless, epistasis analysis and microarray studies reveal that a substantial portion of clade I TGA function is independent of NPR1 (Shearer et al., 2012). Rather, *tga1 tga4* double mutants are compromised in basal resistance against *P. syringae* (Wang and Fobert, 2013; Shearer et al., 2012; Kesarwani et al., 2007; Lindermayr et al., 2010). This phenotype is associated with impaired pathogen-induced callose deposition, oxidative burst, and PR1 protein accumulation which are hallmarks of the response to PAMPs (Wang and Fobert, 2013).

In the flower, BOP1/2 were identified as functioning partner to TGA8/PAN based on genetic interaction studies and a demonstration of complex formation in yeast and Arabidopsis

leaf protoplasts (Hepworth et al. 2005; Xu et al., 2010). Usually, there are 2 carpels, 4 petals, 4 sepals and 6 stamens in a wild-type Arabidopsis flower. In a *bop1 bop2* double mutant, the radial symmetry of flowers is broken by the development of extra organs on the abaxial (ventral) side of the floral meristem. This extra growth results in the development of an adaxial (dorsal) sepal, followed by four sepals that are spaced equally from each other. Furthermore, the abaxial-most pair of sepals are partially turned into petals with outward growth that gives an asymmetric shape to the flower (Hepworth et al., 2005). Sometimes in the *bop1 bop2* mutant, the abaxial side of the floral meristem becomes the new location for an extra stamen to form. The sepal number is regulated through the BOP1/2 and PAN complex as a mutation to PAN can lead to extra abaxial organs with a nearly identical change to a pentamerous state as observed in the *bop1 bop2* double mutant. Other than TGA8, Clade IV TGA9 and TGA10 mediate anther development and interact with CC-type floral glutaredoxins, but their association with BOP1/2 have not been identified (Murmu et al., 2010). Clade I TGA1 and TGA4 regulate Arabidopsis root initiation and root hair density in response to nitrate. A systemic approach revealed that both clade I *TGA1* and *TGA4* were highly induced after nitrate treatment in root and function through directly binding to genes coding transmembrane nitrate carrier during a nitrate response (Alvarez et al., 2014; Canales et al., 2017). The loss-of-function *tgal tga4* mutant exhibits no obvious developmental defects other than a curvature of leaves (Shearer et al., 2012).

1.8 The role of WRKY transcription factors in plant development and defense

WRKY transcription factors comprise one of the largest and diverse protein superfamilies in Arabidopsis. To date, there are more than 70 known WRKY transcription factors in Arabidopsis, many of which have been identified as regulators of plant tolerance to biotic and abiotic stresses.

All WRKY proteins share a highly conserved DNA-binding domain consisting the WRKYGQK peptide sequence and a zinc-finger motif found in all members (Eulgem and Somssich, 2007). In the mid 1990s, first WRKY transcription factor SWEET POTATO FACTOR1 was isolated from *Ipomoea batatas* as a regulator of sporamin and β -amylase synthesis (Ishiguro and Nakamura, 1994). The function of this family was further extended to plant defense when three WRKYs were identified as regulators of *PR1* in parsley (Rushton et al., 1996). Progressively, the functions of WRKY transcription factors have been expanded into almost every aspect of plant biology, including response to abiotic and biotic stress, senescence, development, and secondary metabolites biosynthesis (Chen et al., 2019b; Bakshi and Oelmüller, 2014). WRKY proteins function by binding to a DNA *cis* element designated as the W box (C/TTGACT/C) but variations on this binding site have been reported (Van Verk et al., 2008).

Many aspects of plant growth are regulated by phytohormone signaling pathways, so early studies of WRKY transcription factors were often focused in this area. The function of WRKY proteins became linked to plant defense soon after their discovery (Dong et al., 2003; Jang et al., 2010; Withers and Dong, 2016). Genome-wide analyses of defense responses in *Arabidopsis* have suggested that many key genes activated during plant defense responses are targets of WRKY transcription factors, including *PR1* and *NPRI*. Genome-wide screening has identified many defense-related genes with W-boxes in their promoters (Wang et al., 2006; Eulgem and Somssich, 2007). The functioning mode of WRKY transcription factors is diverse and highly dependent on their binding partners. One well known example is the trimeric complex consisting of WRKY18, WRKY40, and WRKY60. When *WRKY18* was overexpressed alone, the transgenic plants showed more resistance to *P. syringae*. When *WRKY18* was co-expressed with *WRKY40* and *WRKY60*, the transgenic plants suffered from increased susceptibility. Characterization of the phenotypes of

wrky18 wrky40 wrky60 mutant showed enhanced resistance to a hemibiotrophic *P. syringae* microbial pathogen but more susceptibility to a necrotrophic *B. cinerea* fungal pathogen indicating a potential crosslink between SA and JA signaling (Xu et al., 2006).

Despite the broad role of WRKY transcription factors in plant defense and wide investigation on them, it was not until recently that a direct link between NPR1 and WRKY18/70 transcription factors was demonstrated. Biochemical evidence showed that NPR1 interacts with WRKY70 and WRKY18 *in vitro*. In both cases, the interactions between NPR1 and WRKY70/18 were tightly regulated by post-translational modifications of NPR1 protein (Saleh et al., 2015; Chen et al., 2019a). Similar to WRKY18, WRKY70 was shown to have a versatile yet opposite role in plant defense, depending on its binding partners. In the absence of infection, WRKY70 represses many pathogen-inducible genes, including *SARD1*, to prevent unnecessary defense responses (Zhou et al., 2018). During a defense response, WRKY70 becomes a positive regulator of defense marker genes such as *PR2* and *PR5* under SA signaling but a repressor of JA-inducible genes (Li et al., 2004).

1.9 Thesis rationale

In summary, BOP1 and BOP2 are paralogs of the Arabidopsis defense regulator NPR1. Although BOP1/2 and NPR1 share a similar domain structure, their *in planta* roles are significantly different. NPR1 has been identified as a central hub in SA-mediated defense responses and its activity is heavily controlled primarily by post-translational regulations. On the other hand, BOP1 and BOP2 are plant development regulators with an enriched expression at organ boundaries where cell growth is restricted. To date, functional analysis of BOP1 and BOP2 has focused on characterization of loss-of-function *bop1 bop2* phenotypes in controlling SAM activity and plant

architecture. BOP1/2 function as transcriptional co-factors but only one regulatory partner was identified, TGA8/PAN. Given that *pan* and *bop1 bop2* genetic interactions are confined to developing flowers, we speculated that other TGA partners mediate broader functions of BOP1/2 in development and possibly defense. A microarray study identified an upregulation of *TGA3*, *TGA4*, and *TGA9* in plants overexpressing *BOP1* suggesting the possible involvement of these TGAs in BOP-dependent processes. My thesis specifically examines a potential role for clade I TGA1 and TGA4 with BOP1/2 in development and defense.

1.10 Hypothesis

- (a) BOP1/2 interact with clade I TGAs for the transcriptional regulation of genes involved in boundary development and plant architecture.
- (b) BOP1/2 function in the same genetic pathway as clade I TGAs in regulation of plant defense.

1.11 Major objectives

The major experimental objectives of my work were as follows:

- 1) To test if clade I TGAs function as co-factors for BOP1/2 in regulating organ boundary processes.
- 2) To test if BOP1/2 contribute to plant defense, and if so:
 - a) does this defense role require clade I TGA factors?
 - b) does this defense role require NPR1?

Figure 1.1 Organization of the SAM and architecture of model plant *Arabidopsis thaliana*.

The development of plant architecture is attributed to changes in the activity of the shoot apical meristem (SAM) over the lifespan. Stem cells in the SAM constantly divide to provide founder cells for all of the aerial tissues in a plant. To maintain the growth of a shoot, reprogramming occurs as cell position within the SAM changes. Lateral organs (such as leaf primordia) that emerge on the flanks of the meristem are rapidly separated from the meristem by boundaries (highlighted in pink). Boundaries are zones of repressed growth required in separating differentiating organs from the meristem and from their adjacent organs as they form. During the vegetative stage, the SAM produces rosette leaves in a spiral pattern with dormant axillary meristems and no stem elongation. The transition from a vegetative stage to a reproductive stage is caused by floral inductive signals that act on the SAM. These signals cause the meristem to adopt a reproductive, inflorescence meristem (IM) fate. This change in fate results in new patterns of meristem activity that produce an inflorescence. During the vegetative stage, floral repressors in the SAM block meristem competence to flowering. A variety of pathways that monitor environmental, nutrient, and age-related signals control the abundance of floral repressors and factors that promote IM and floral meristem identity to control flowering time.

Figure 1.2 Interactions of BOP1/2 with TALE homeobox genes in the regulation of Arabidopsis flowering and inflorescence architecture.

(A) Drawing of an Arabidopsis shoot apex. The shoot apical meristem (SAM) is shown in grey. Homeobox genes *PNY* and *PNF* maintain SAM integrity by containing the activity of boundary genes to the base of developing organs. The defects in *pny* and *pny pnf* mutants are caused by misexpression of a set of boundary genes including *BOP1* and *BOP2*. (B) An inflorescence from a wild-type plant. (C) An inflorescence from a *pny* mutant showing abnormal clustered siliques caused by defects in the pattern of organ initiation and internode elongation. (D) The *pny pnf* mutant cannot flower; (E) Mutation of *BOP1/2* corrects inflorescence architecture defects in a *pny* mutant. (F) Mutation of *BOP1/2* restores flowering in a *pny pnf* mutant. (G) Simplified regulatory mechanism showing interactions between *BOP1/2* and TALE homeobox genes of the meristem and boundary maintains meristem activity required for flowering. PNY and PNF proteins repress *BOP1/2* and boundary-related TALE homeobox genes *KNAT6* and *ATH1*. This repression of boundary genes in the meristem is required for flowering.

Figure 1.3 Schematic illustration of flg22-triggered PTI response in Arabidopsis.

Pattern-triggered immunity (PTI) starts from the perception of conserved pathogen-associated molecular patterns (PAMPs) from microbes. This scheme shows a flg22-induced PTI response in Arabidopsis. FLS2 is a cell surface-anchored pattern-recognition receptor which can detect such molecular patterns and initiate PTI. Activation of FLS2 by flg22 stimulates an interaction with the leucine-rich receptor-like kinase BAK1 and cytoplasmic kinase BIK1 resulting in phosphorylation (labeled as “P” in red circles) of BIK1. Two major events after perception of flg22 are: the production of reactive oxygen species (ROS) by the NADPH oxidase RBOHD at the plasma membrane and the initiation of signaling passed through mitogen activated protein kinase (MAPK) cascades. Both events lead to the activation of transcription factors (TFs) that regulate downstream defense-related gene expression.

Figure 1.4 A comparison between NPR1 and BOP1/2 gene structure

A comparison between NPR1 and BOP1/2. Coding region are shown in boxes. Protein domains with known functions are differed with various patterns: boxes with dots represent BTB/POZ domains; boxes with vertical lines show ankyrin-repeat domains; box with diagonal stripes shows a nuclear localization sequence.

CHAPTER 2

Clade I TGA bZIP transcription factors mediate BLADE-ON-PETIOLE-dependent regulation of development in *Arabidopsis thaliana*

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2.1 Abstract

Lateral organs formed by the shoot apical meristem (SAM) are separated from surrounding stem cells by regions of low growth called boundaries. *Arabidopsis* (*Arabidopsis thaliana*) *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2* represent a class of genes important for boundary patterning in land plants. Members of this family lack a DNA-binding domain and interact with TGACG-motif binding (TGA) basic leucine zipper (bZIP) transcription factors for recruitment to DNA. Here, we show that clade I bZIP transcription factors TGA1 and TGA4, previously associated with plant defense, are essential co-factors in BOP-dependent regulation of development. *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. Further, we show that clade I TGAs interact constitutively with BOP1 and BOP2, contributing to activation of *ARABIDOPSIS THALIANA HOMEBOX GENE1*, which is needed for boundary establishment. These studies expand the functional repertoire of clade I TGA factors in development and defense.

2.2 Introduction

The shoot apical meristem (SAM) provides all of the aerial structures in a plant. This primary meristem is a constant source of cells for the production of leaves, stems, branches, and flowers throughout the life cycle (Aichinger et al., 2012). Lateral organs formed by the SAM are separated from the stem cell domain by low-growth regions called boundaries. Impairment of boundaries leads to SAM termination and/or fusion of organs (Aida and Tasaka, 2006b, 2006a). As organs enlarge, boundaries define the base of shoots and control specific aspects of plant architecture. Boundaries provide axillary meristems essential for flower formation and branching. Boundaries are also sites for the regulated detachment of organs from the plant body, which is essential for abscission and seed dispersal (Hepworth and Pautot, 2015).

BLADE-ON-PETIOLE (BOP) genes, first described in *Arabidopsis* (*Arabidopsis thaliana*), are regulators of boundary patterning in land plants (Khan et al., 2014). BOP1 and BOP2 encode closely-related transcriptional co-activators characterized by an N-terminal BTB/POZ (Broad-Complex, Tramtrack, and Bric-a-brac/POX virus and zinc finger) domain located upstream of an ankyrin repeat domain (Chan et al., 2004; Hepworth et al., 2005; Norberg et al., 2005). The BTB/POZ domain interacts with CULLIN3-RING E3 ubiquitin ligase to target transcription factors for degradation (Fu et al., 2012; Zhang et al., 2017; Chahtane et al., 2018). The ankyrin repeats interact with TGA (TGACG-motif binding) basic region/leucine zipper motif (bZIP) transcription factors for recruitment to DNA (Zhang et al., 1999; Despres et al., 2000; Zhou et al., 2000; Hepworth et al., 2005; Xu et al., 2010). In *Arabidopsis*, BOP1/2 are part of a larger family of six BTB-ankyrin proteins. This family contains two phylogenetic subclades: a primary subclade comprised of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) and three

close homologs (NPR2, NPR3, and NPR4) with roles in plant defense and a secondary subclade comprised of BOP1 and BOP2 with roles in plant development (Khan et al., 2014). NPR1 is essential for a broad-spectrum defense response called systemic acquired resistance (SAR) mediated by salicylic acid (SA). NPR3 and NPR4 also play a role in this response (Cao et al., 1997; Fu et al., 2012; Wu et al., 2012a; Yan and Dong, 2014; Ding et al., 2018). By contrast, BOP1/2 are global regulators of plant architecture. In aerial parts of the plant, they act at lateral organ boundaries to regulate abscission and the morphogenesis of leaves, inflorescences, fruits, and flowers (Khan et al., 2014; Hepworth and 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). Despite these functional differences, NPR1 and BOP1/2 share a highly conserved domain structure, suggesting a similar mode of action (Khan et al., 2014).

In particular, BTB-ankyrin proteins lack a DNA-binding domain and interact with transcription factors for recruitment to DNA. Yeast-two hybrid screens have been used to identify TGA bZIP transcription factors important for NPR1 function (Zhang et al., 1999; Despres et al., 2000; Zhou et al., 2000; Després et al., 2003; Boyle et al., 2009). TGA transcription factors in *Arabidopsis* are divided into five subclades (clade I: TGA1 and TGA4; clade II: TGA2, 5, and 6; clade III: TGA3 and TGA7; clade IV: TGA9 and TGA10; and clade V: TGA8, also known as PERIANTHIA/PAN) (Gatz, 2013). Genetic studies show highly overlapping roles for TGA factors in plant defense, stress responses, and/or development (Gatz, 2013; Noshi et al., 2016). NPR1 constitutive interaction with clade II TGAs plays a major role since SAR is abolished in *tga2 tga5 tga6* triple mutants, resulting in an *npr1*-like phenotype (Cao Hui et al., 1994; Zhang et al., 2003).

NPR1 also interacts with clade III TGA3 (Choi et al., 2010) and in a redox-dependent manner with clade I TGA1 and TGA4 to impart its function (Després et al., 2003; Sun et al., 2018).

In previous work, we showed that BOP1/2 interact with clade V TGA8/PERIANTHIA (PAN). Loss-of-function *bop1 bop2* and *pan* mutants have a similar pentameric arrangement of first-whorl floral organs and extra petals, showing that BOP-PAN interactions regulate floral patterning (Hepworth et al., 2005; Xu et al., 2010). However, inactivation of TGA8/PAN captures only a subset of *bop1 bop2* loss-of-function phenotypes, suggesting the involvement of other TGA family members (Running and Meyerowitz, 1996; Chan et al., 2004; Hepworth et al., 2005; Norberg et al., 2005; Xu et al., 2010; McKim et al., 2008).

Here, we identify a role for clade I TGAs in BOP1/2-dependent regulation of plant architecture. These TGA factors are co-expressed at organ boundaries and function in the same genetic pathways as BOP1/2 important for meristem integrity, flowering, and inflorescence architecture. Distinct from NPR1, TGA1 and TGA4 interact constitutively with BOP1/2 in yeast (*Saccharomyces cerevisiae*) cells. Further, clade I TGAs and BOP1 co-localize to A-box-containing sites in the promoter of boundary gene *ARABIDOPSIS THALIANA HOMEBOX GENE1* (*ATH1*), contributing to its activation. These results highlight a dual role for clade I TGAs in development and defense through differential interaction with BTB-ankyrin proteins.

2.3 Materials and methods

2.3.1 Plant materials and growth conditions

Seeds were germinated *in vitro* on minimal media (Haughn and Somerville, 1986). Seedlings were transferred to sterilized soil (Promix BX Black, Premier Horticulture) supplemented with a 0.1%

(w/v) solution of 20-20-20 fertilizer. Plants were grown to maturity in growth chambers at 21°C with light intensity of 110-130 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under short days (8 hours light/16 hours dark), long days (16 hours light/8 hours dark) or continuous light (24 hours light) as required. Wild type in this study was the Col-0 ecotype of *Arabidopsis* (*Arabidopsis thaliana*). *35S:BOP2* and *bop1-6D* (re-named as *BOP1-oe* in this thesis) overexpression lines were described previously (Norberg et al., 2005). Mutant lines were obtained from the Arabidopsis Biological Resource Center (<https://abrc.osu.edu/>). The *bop1-3* (SALK_012994), *bop2-1* (SALK_075879), *tga1-1* (SALK_028212), *tga4-1* (SALK_127923), *pnf-40126* (SALK_40126) and *pnf-96115* (SALK_96116) alleles have been described previously (Smith and Hake, 2003; Smith et al., 2004; Hepworth et al., 2005; Kesarwani et al., 2007). All mutant combinations were generated by crossing and confirmed by genotyping (Smith and Hake, 2003; Smith et al., 2004; Hepworth et al., 2005; Devi, 2014). *BOP1pro:GUS* and *BOP2pro:GUS* reporter lines have been previously described (McKim et al., 2008; Xu et al., 2010). Primers used in this study are listed in Table 2.1. Constructs were used to transform plants by floral dipping (Clough and Bent, 1998) using the *Agrobacterium tumefaciens* strain C58C1 GV101 pMP90 (Koncz and Schell, 1986).

2.3.2 Construction of GUS reporter gene lines and staining

To create *TGA1pro:GUS* and *TGA4pro:GUS* reporter genes, the 5' untranslated region up to and including the start codon for *TGA1* (nt -3969 to +3) and *TGA4* (nt -3950 to +3) were amplified from genomic DNA as template [BAC M1QN23 for *TGA1* and BAC T31P16 for *TGA4*] using iProof DNA polymerase (Bio-Rad). Primers incorporating *Bam*HI and *Nco*I restriction sites at the 5' ends were used to facilitate directional cloning. Products were cloned into pCR-BluntII-TOPO (Invitrogen) and sequenced. Inserts were released by digestion with *Bam*HI and *Nco*I and ligated

into the corresponding sites of pGCO:GUS to create translational fusions with the GUS gene *uidA* (Hepworth et al., 2002). *A. tumefaciens* was co-transformed with pSOUP and introduced into wild-type plants (Hellens et al., 2000). Glufosinate-ammonium resistant primary transformants were selected on soil. Homozygous lines were isolated for downstream experiments. Tissues were stained and/or embedded and sectioned for GUS activity as described (Woerlen et al., 2017). Low resolution images were collected under bright field using a stereomicroscope (SteRIO Discover V20, Carl Zeiss). A compound microscope (Axio Imager M2, Carl Zeiss) was used for high-resolution images.

The construction of 2-kb *ATH1pro:GUS D35S:BOP1-GR* transgenic plants was previously described. This line contains a steroid-inducible form of BOP1 that upregulates expression of a 2-kb *ATH1pro:GUS* reporter gene in the presence of DEX (Khan et al., 2015). Transgenes were introduced by crossing into *tgal tga4* double mutant background. F1 and F2 progenies were grown on minimal media using 25 $\mu\text{g ml}^{-1}$ hygromycin B and 10 $\mu\text{g ml}^{-1}$ phosphinothricin for selection of *D35S:BOP1-GR* and 2-kb *ATH1pro:GUS*, respectively. Mutants homozygous for *tgal* and *tga4* were identified by genotyping in the F2 generation (Shearer et al., 2012).

For analysis of GUS activity, homozygous 2-kb *ATH1pro:GUS D35S:BOP1-GR tgal tga4* plants were germinated on minimal selection media containing 3 μM DEX under continuous light. After a week, seedlings were moved to soil and treated daily with a 10 μM DEX solution (prepared from a 30 mM stock solution in ethanol). A 0.12% ethanol solution (v/v) was used for mock treatment. The inflorescence apices from 30-day-old plants were harvested and stained for GUS activity.

2.3.3 Yeast two-hybrid assay

Protein-protein interactions were assayed using a Matchmaker® GAL4-based yeast two-hybrid system (Clontech) and Gateway-compatible pGPKT7-DEST (bait) and pGADT7-DEST (prey) plasmids modified from pGBKT7 and pGADT7-Res vectors, respectively (Lu et al., 2010). BOP1 and BOP2 proteins fused to the DNA-binding domain of yeast GAL4 were used as bait. TGA1, TGA4, and TGA8/PAN proteins fused to the transcriptional activation domain of yeast GAL4 were used as prey. To prepare Gateway entry vectors, the full-length coding sequences of *BOP1*, *BOP2*, *PAN*, *TGA1*, and *TGA4* were cloned into pCR8/GW/TOPO-TA (ThermoFisher Scientific). Template plasmids containing mutated forms of TGA1 (mTGA1/C260N/C266S) and TGA4 (mTGA4/C256S/C262S) were supplied by Charles Després (Després et al., 2003). The full-length coding sequences of *mTGA1* and *mTGA4* were cloned into pENTR/D-TOPO (ThermoFisher Scientific). All entry vector inserts were sequenced to confirm authenticity. To make the final plasmids, LR reactions were performed using Gateway LR Clonase II enzyme mix according to the manufacturer's instructions (Invitrogen). Bait and prey plasmid were co-transformed into yeast AH109 strain using a high-efficiency lithium acetate method (Gietz and Schiestl, 2007). Yeast transformed with bait and prey plasmids were identified by selection on synthetic defined (SD) media plates lacking leucine and tryptophan (SD/-Leu/-Trp). Positive colonies were cultured in fresh SD/-Leu/-Trp liquid medium. Dilution series were spotted onto SD/-Leu/-Trp medium or SD medium lacking leucine, tryptophan, and histidine plus 8 mM 3-AT (3-amino-1,2,4-triazole, Sigma-Aldrich) (SD/-Leu/-Trp/-His/+3-AT) for assessment of histidine reporter gene activity. A 3-AT concentration (8 mM) sufficient to distinguish positive growth from background was determined empirically. Dilution series were spotted in replicate onto SD/-Trp/-Leu/-His/+3-AT

media supplemented with 20 mg l⁻¹ of X- α -Gal (Clontech) for assessment of α -galactosidase reporter gene activity.

2.3.4 Bimolecular fluorescence complementation (BiFC) assay

Protein-protein interactions *in planta* were analyzed using a Gateway-compatible BiFC system (Tsai and Gazzarrini, 2012). Donor vectors for yeast two-hybrid were used for LR recombination into BiFC vectors to obtain *nYFP-BOP1*, *nYFP-BOP2*, *cYFP-PAN*, *cYFP-TGA1*, and *cYFP-TGA4* fusions, respectively. BiFC constructs were introduced into *A. tumefaciens* GV3101 by electroporation. Leaves of six-week-old *N. benthamiana* plants were infiltrated as described previously (Li et al., 2009). Samples were imaged using a Carl Zeiss LSM800 Airyscan confocal laser scanning microscope four days after infiltration. Nuclei were stained by incubating leaf discs in distilled water containing 5 μ g ml⁻¹ of the reagent 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Afterwards, leaf discs were mounted on a microscope slide for observation through the abaxial side. For visualization of YFP, chlorophyll *a*, and DAPI fluorescence, excitation lasers of 488 nm, 488 nm, and 405 nm were used, respectively. Emission bands of 410-605 nm for YFP, 650-700 nm for chlorophyll *a*, and 410-605 nm for DAPI were acquired using gallium-arsenide-phosphide detectors and a Plan-Apo 40x/1.4 oil objective. Images were processed using Zen 2.3 software (Carl Zeiss).

2.3.5 Construction of *TGA1pro:TGA1-GFP* and *TGA4pro:TGA4-GFP tga1 tga4* transgenic plants

ChIP-qPCR assays were used to evaluate binding of TGA1 and TGA4 proteins to A-box sites in the *ATH1* promoter, which was previously identified as enriched for BOP1 (Khan et al., 2015).

For this, translational fusions to GFP (green fluorescent protein) were generated to allow use of commercially available anti-GFP antibody for ChIP. Translational fusions were expressed behind the same promoters as used for GUS lines. To create *TGA1pro:TGA1-GFP*, a genomic DNA fragment including the 5' untranslated and coding region of *TGA1* without a stop codon (nt -4122 to +1716) was amplified from BAC DNA using iProof as the polymerase (BioRad). The resulting fragment was cloned into Gateway®-compatible entry vector pENTR/D-TOPO (Invitrogen). The insert was sequenced to ensure fidelity. Translational fusion with GFP was achieved by moving the *TGA1pro:TGA1* insert into destination vector pMDC107 (Curtis and Grossniklaus, 2003) using LR Clonase (Invitrogen). After LR recombination, the junction between *TGA1* and *GFP* was sequenced to ensure the fusion was in-frame. To create *TGA4pro:TGA4-GFP*, DNA sequences containing the putative *TGA4* promoter and coding sequences of *TGA4* and *GFP* were joined together in the binary vector pBAR1 (Holt III et al., 2002) by Gibson assembly (Gibson et al., 2009). The 5' untranslated sequence of *TGA4* (nt – 3949 to +0) was amplified from BAC DNA. The coding sequence of *TGA4* without a stop codon was amplified from cloned cDNA template. The *GFP* coding sequence was amplified from pMDC107. *TGA4* and *GFP* coding sequences were first assembled in pBAR1 linearized with *Xba*I using In-Fusion (Clontech). The resulting plasmid was linearized with *Bam*HI for incorporation of *TGA4pro* sequence resulting in the final construct. The assembled inserts were sequenced to ensure fidelity.

Constructs were introduced into *tgal tga4* mutant plants. *TGA1pro:TGA1-GFP* primary transformants were selected on agar plates containing 25 mg ml⁻¹ hygromycin B. *TGA4pro:TGA4-GFP* primary transformants were selected on agar plates containing 10 mg ml⁻¹ phosphinothricin. Homozygous lines were generated for downstream experiments.

To monitor the subcellular localization of fusion proteins, transgenic seedlings were imaged under a Carl Zeiss LSM800 Airyscan confocal laser scanning microscope. For visualization of GFP and chlorophyll *a*, excitation lasers of 488 nm were used. Emission bands of 500-617 nm for GFP and 645-700 nm for chlorophyll *a* were acquired using gallium-arsenide-phosphide detectors and a Fluor 20x/0.75 M27 objective. Images were processed with ZEN 2.3 software (Carl Zeiss).

2.3.6 Tunicamycin assays

Surface-sterilized seeds were germinated on 0.5X Murashige and Skoog (MS) medium containing tunicamycin (TM) (Sigma). Five-day-old seedlings were immersed into liquid 0.5X MS media with or without 0.8 $\mu\text{g ml}^{-1}$ TM for six hours. After treatment, the seedlings were rinsed three times with liquid 0.5X MS media and then recovered for five days on fresh 0.5X MS plates. Fresh weight of seedlings (n=5 per treatment group, 5-6 replicates) were measured and quantified essentially as described (Wang and Fobert, 2013). The experiment was repeated twice with similar results.

2.3.7 Chromatin Immunoprecipitation (ChIP) assay

The ChIP assay was performed according to a previously described protocol (Gendrel et al., 2005) using an anti-GFP antibody (Ab290, Abcam). Homozygous *TGA1pro:TGA1-GFP tga1 tga4*, *TGA4pro:TGA4-GFP tga1 tga4*, and *tga1 tga4* seeds were germinated on minimal media under long days and transferred to soil for continued growth. Apex tissue was collected from 4-week-old flowering plants for analysis. Quantification of immunoprecipitated DNA by RT-qPCR was performed. The input % of each primer set was first obtained by comparing Ct value with input DNA. The Ct value using *UBIQUITIN5* promoter (Boyle et al., 2009) was used to normalize data.

Final results were shown relative to the *tga1 tga4* genetic control. Primers used for ChIP-qPCR are listed in Table 2.1.

2.3.8 RT-qPCR

Total RNA was isolated from seedlings, apices, or internodes using Trizol reagent (Invitrogen). Plant seeds were germinated on minimal media plates under long days. Ten-day-old seedlings were harvested for total RNA isolation. For apices, five-day-old seedlings were transferred onto soil for continued growth under long days. Dissected apices were <0.5 cm tall with the majority of flowers under stage 13. For internodes, a fresh razor blade was used to harvest tissue from the primary and secondary inflorescences of 5-6 plants, starting above the first silique from the bottom and all the way to where the internodes were too small (less than 0.2 cm long) to collect.

For internodes, cDNA was synthesized using 1 µg of RNA and RT-qPCR was performed as previously described (Khan et al., 2012a). For seedlings and apex, RNA samples were treated with Turbo DNase I (Ambion) at 37°C for 30 min followed by treatment with DNase Inactivation Reagent (Ambion) as instructed by the manufacturer. Reverse transcription was performed using 1 µg of total RNA and SuperScript III Reverse Transcriptase (Invitrogen) with Oligo d(T) according to manufacturer's instruction. Quantitative PCR was carried out using 2 µl of 10-fold diluted cDNA, gene specific primers (Table 2.1), and Power SYBR Green PCR MasterMix (Applied Biosystems) on a StepOnePlus Thermocycler (Applied Biosystems). Results were calculated using the ΔC_t method (Pfaffl, 2001). The arithmetic means of *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT (GAPC)*, *TUBULIN BETA CHAIN 4 (TUB4)*, and *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1 (EIF4A1)* were used as

normalization controls. At least two technical replicates were performed for each sample. Data shown are the average of three independent biological replicates. Error bars, standard error.

2.3.9 Accession numbers

Sequence data from this article is available in the EMBL/Genbank data libraries under accession numbers: At5g02030 (*PNY*), At2g27990 (*PNF*), At3g57130 (*BOP1*), At2g41370 (*BOP2*), At1g68640 (*PAN*), At5g65210 (*TGAI*), At5g10030 (*TGA4*), At4g32980 (*ATH1*), At3g04120 (*GAPC*), At5g44340 (*TUB4*), At3g13920 (*EIF4A1*), and At3g62250 (*UBQ5*).

2.4 Results

2.4.1 Transcript profiling identifies clade I TGAs upregulated by BOP1

TGA bZIP transcription factors are required for the nuclear functions of BOP1/2 (Khan et al., 2014). The TGA family contains 10 members (Gatz, 2013). To identify potential partners, we used the transcriptome of *BOP1*-overexpressing plants to determine co-regulated *TGA* bZIP genes (Khan et al., 2015). Within our microarray dataset, *TGA4* transcripts were upregulated 3.1-fold relative to wild type. These data were validated by RT-qPCR experiments showing that both clade I *TGAI* and *TGA4* transcripts were significantly increased in the stem of *BOP1-oe* transgenic plants that overexpress *BOP1* (Figure 2.1A). Despite this increase, these genes are not direct targets of BOP1 (Khan, 2013). Loss-of-function *bop1 bop2* mutations disrupt organ boundary patterning resulting in loss of abscission, leafy petioles, fused organs, and characteristic defects in floral patterning (Khan et al., 2014). Consistent with previous reports, no such boundary-related defects have been observed in *tgal*, *tga4*, or *tgal tga4* mutants (Kesarwani et al., 2007; Shearer et al., 2012; Alvarez et al., 2014). The only obvious similarity to *bop1 bop2* mutants is “pinwheel”

curvature of leaves in *tgal* and *tgal tga4* double mutants (Figure 2.1B; see also Shearer et al., 2012).

2.4.2 TGA1/4 are required by BOP1/2 to exert changes in inflorescence architecture

To reveal a potential role for clade I TGAs, we studied the impact of *tgal* and *tga4* loss-of-function mutations on the phenotype of *35S:BOP2* transgenic plants. Constitutive overexpression of *BOP1* or *BOP2* inhibits stem elongation, resulting in short plants (Norberg et al., 2005; Khan et al., 2012a, 2012b, 2015). Previously, we showed that this dwarf phenotype is reversed by inactivating boundary genes such as *ATH1* or *KNOTTED1-LIKE FROM ARABIDOPSIS THALIANA6* (*KNAT6*), which function genetically downstream of *BOP1/2* (Norberg et al., 2005; Khan et al., 2012b, 2012a). To test if clade I TGAs are part of this module, *35S:BOP2* plants were crossed with wild-type control plants, *tgal* or *tga4* single mutants, and *tgal tga4* double mutants. The F1 progenies of *35S:BOP2* plants crossed to wild-type and *tgal* or *tga4* single mutant plants were dwarf. By contrast, the F1 progenies of *35S:BOP2* plants crossed to *tgal tga4* double mutant plants showed recovery of stem elongation. Approximately 82% of plants in this population (155/190) were tall (≥ 12 cm high) (Figure 2.2). These data suggest that TGA1 and TGA4 are required for BOP1/2-dependent changes in inflorescence architecture.

2.4.3 Clade I TGAs function in the same genetic pathways as BOP1/2

SAM integrity relies on a precise balance between maintenance of the stem cell population and organogenesis (Aichinger et al., 2012). Two closely related BELL homeodomain proteins, PENNYWISE (PNY) and POUND-FOOLISH (PNF), required for meristem integrity enable SAM maintenance and flowering by spatially regulating boundary genes, such as *BOP1/2*, in the shoot

apex and stem (Smith et al., 2004; Khan et al., 2012b, 2015; Bencivenga et al., 2016). In *pnf* mutants, apical dominance is partially lost and irregular stem elongation results in inflorescences with clusters of flowers/silques instead of even spacing (Byrne et al., 2003; Smith and Hake, 2003). In *pnf pnf* double mutants, the SAM is narrow and terminates prematurely in a small majority of seedlings (Ung and Smith, 2011; Ung et al., 2011). Further, these mutants are incapable of elongating a stem and fail to produce flowers (Smith et al., 2004). Inactivation of *BOP1/2* or genes acting downstream of *BOP1/2* complements *pnf* and *pnf pnf* mutant phenotypes (Khan et al., 2012b, 2012a, 2015). This complementation occurs because repression of boundary genes by PNF and PNF preserves organized division of cells in the SAM required for meristem function, including the production of stem and flowers (Ung et al., 2011; Bencivenga et al., 2016; Andrés et al., 2015).

To examine if clade I *TGAs* function in the same genetic pathway, we crossed *pnf* and *pnf pnf* mutants with *tga1*, *tga4*, and *tga1 tga4* mutants and analyzed the phenotypes of the resulting higher-order mutants. SAM termination defects are a characteristic of *pnf pnf* mutations. Figure 2.3 shows that approximately 48.2% of *pnf pnf* mutants stopped making leaves, indicating failed meristem activity. No such arrest was observed in *tga1 pnf pnf*, *tga4 pnf pnf*, and *tga1 tga4 pnf pnf* mutants, indicating that inactivation of clade I *TGAs* restores meristem function. We also tested if *pnf* and *pnf pnf* defects in flowering and inflorescence architecture could be rescued. Figure 2.4 shows that *pnf* silique clustering defects were partially rescued by *tga1* and completely rescued by *tga1 tga4* mutations. Further, flowering in *pnf pnf* was restored by *tga1*, *tga4*, and *tga1 tga4* mutations. Quantitative phenotypic analyses showed that parameters of inflorescence architecture and flowering time in rescued genotypes were similar to wild type (Supplemental Figure S2.1).

These collective data suggest that clade I *TGAs* function as boundary genes acting in the same genetic pathway as *BOP1/2* to control meristem function and inflorescence architecture.

2.4.4 TGA1 and TGA4 expression is enriched at organ boundaries

BOP1 and *BOP2* expression is absent from the SAM and enriched at organ boundaries throughout the life cycle (Chan et al., 2004; Norberg et al., 2005; Xu et al., 2010; Khan et al., 2012b; Andrés et al., 2015; Khan et al., 2015). To examine if *TGA1* and *TGA4* are also expressed in organ boundaries, we used promoter fusions to β -glucuronidase (GUS) (Jefferson et al., 1987). Analysis of stained tissue showed that *TGA1* and *TGA4* were expressed in roots, leaves, stems, and flowers (Figure 2.5). This distribution matches *in silico* gene expression profiling data from AtGenExpress (Schmid et al., 2005; Waese et al., 2017). Within aerial tissues, *TGA1* and *TGA4* expression was enriched in the vasculature of leaves and stems. Cross-sections of the primary stem showed that *TGA4* was expressed in phloem and both genes were expressed in xylem, whereas *BOP1* and *BOP2* expression was largely absent from internodes (Figure 2.5). Comparing to *BOP1* and *BOP2*, strong overlap with *TGA1* and *TGA4* expression was observed at the base of young leaves and in vasculature at the base of mature rosette leaves. Strong overlap in expression was also observed at shoot and organ boundaries in the inflorescence, including nodes where floral pedicels join to the stem, the base of young floral organs where abscission zones form, and the base of mature fruits where a protective layer is deposited post-abscission (Figure 2.5). These data indicate a boundary-enriched expression pattern for clade I *TGAs* that partially overlaps with *BOP1* and *BOP2*. Similar to other boundary genes, no *TGA1* or *TGA4* expression was detected in the SAM (Supplemental Figure S2.2).

2.4.5 PNY and PNF repress clade I TGA transcription factor gene expression

PNY and presumably PNF directly repress boundary genes in the SAM and stem to preserve organized division of cells required for pattern formation (Andrés et al., 2015; Bencivenga et al., 2016). Boundary genes including *BOP1/2*, *ATH1*, *KNAT2*, and *KNAT6* have an expanded domain of expression in the stem and apices of *pnv* and *pnv pnf* mutants (Ragni et al., 2008; Khan et al., 2012b, 2012a; Andrés et al., 2015; Khan et al., 2015). This prompted us to test if *TGA1* and/or *TGA4* expression domains are likewise expanded in *pnv* and *pnv pnf* mutants. In *pnv* and *pnv pnf/+* mutants, both genes showed strongly upregulated expression in the stem, particularly around nodes with clustered flowers (Figure 2.6). This pattern was similar to misexpression of *BOP1/2* in *pnv* stems (Khan et al., 2012a). We did not observe misexpression of either gene in the SAM or inflorescence meristem of *pnv pnf* mutants, suggesting that control elements for expression might be located in the introns or 3'UTR that were missing from this experiment (Supplemental Figure S2.2). Overall, these data confirm that PNY and PNF spatially control clade I *TGA* expression, similar to other boundary genes.

2.4.6 Clade I bZIP TGAs are required for activation of *ATH1* expression

BOP1/2 directly activate boundary genes to control leaf patterning and inflorescence architecture (Jun et al., 2010; Khan et al., 2015). Experiments using a steroid-inducible form of BOP1 fused to the hormone-binding domain of the rat (*Rattus norvegicus*) glucocorticoid receptor (BOP1-GR) have shown that BOP1 directly activates *ATH1*, a homeobox gene important for boundary patterning (Gómez-Mena and Sablowski, 2008; Khan et al., 2015). Analysis identified a 2.0-kb fragment of the *ATH1* promoter fused to GUS that is responsive to BOP1 induction. This reporter

gene is up-regulated in *D35S:BOP1-GR* plants after four hours of dexamethasone (DEX) treatment. Stronger induction occurs after 24 hours of sustained DEX treatment (Khan et al., 2015).

To test if *ATH1* expression is likewise dependent on clade I TGAs, we first monitored *ATH1* transcript levels in *bop1 bop2* and *tga1 tga4* double and *bop1 bop2 tga1 tga4* quadruple mutants compared to wild-type control plants (Figure 2.7AB). In seedlings and inflorescence apices, *ATH1* transcripts were significantly reduced in *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* mutants compared to wild-type, which is consistent with these genes functioning in the same genetic pathway. To further test if BOP1 requires TGA1/4 transcription factors to activate *ATH1* expression, *D35S:BOP1-GR* transgenic plants were used to monitor the DEX-induction of a 2.0-kb *ATH1pro:GUS* reporter gene in wild-type versus *tga1 tga4* mutant plants (Figure 2.7C-G). Reporter gene expression was diminished in *tga1 tga4* mutant plants compared to wild type, and no significant induction was observed in most lines after long-term treatment with DEX. DEX-treated *D35S:BOP1-GR tga1 tga4* plants were also significantly taller than *D35S:BOP1-GR* DEX-treated control plants, which is consistent with a requirement for ATH1 in BOP1-dependent restriction of stem elongation (Figure 2.7H; Khan et al., 2012a). Collectively, these data suggest that clade I TGAs are positive regulators of *ATH1* expression.

2.4.7 Clade I TGA factors interact constitutively with BOP1/2

TGA bZIP transcription factors selectively interact with BTB-ankyrin proteins, which can function as transcriptional activators when localized to DNA (Despres et al., 2000; Hepworth et al., 2005; Xu et al., 2010; Wu et al., 2012a). BOP1/2 interactions with clade I TGA1 and TGA4 were previously tested in yeast, but the results were inconclusive (Hepworth et al., 2005). On the other hand, NPR1 selectively interacts with TGA1 *in planta* under reducing conditions triggered by

exposure to SA (Després et al., 2003). This exposure is thought to alter the redox status of TGA1 Cys residues creating a conformational change that brings about interaction with NPR1. mTGA1C260N/C266S mutants (and equivalent mutants of TGA4) are thought to interact constitutively with NPR1 because formation of an inhibitory intramolecular disulphide bridge is abolished (Després et al., 2003).

Yeast two-hybrid is a highly sensitive assay for detecting protein-protein interactions. Therefore, we used this method to reassess BOP interactions with clade I TGA factors using PAN as a positive control. BOP1 and BOP2 fused to the DNA-binding domain of yeast GAL4 were used as bait. Wild-type or Cys mutant forms of TGA1 and TGA4 fused to the activation domain of GAL4 were used as prey. Figure 2.8A shows that BOP1 and BOP2 preferentially interact with wild-type TGA1 and TGA4 in yeast. Interestingly, redox mimic mutations that promote TGA1 (C260N/C266S) and TGA4 (C256S/C262S) interaction with NPR1 reduce interactions with BOP1 and BOP2. As a further test, we used bimolecular fluorescence complementation (BiFC) to examine BOP1 and BOP2 interaction with TGA1 and TGA4 *in planta*. Figure 2.8B shows reconstitution of yellow fluorescent protein (YFP) fluorescence in nuclei of transformed *Nicotiana benthamiana* leaf cells, indicating that BOP1 and BOP2 form a complex with TGA1 and TGA4 in plant cells.

2.4.8 Clade I bZIP TGAs directly bind to the ATH1 promoter

An interaction between BOP1/2 and clade I TGAs in nuclei suggests that these proteins might co-localize to the promoter of target genes to activate transcription. The *ATH1* promoter contains abundant motifs that match or closely match consensus TGA binding sites (Khan et al., 2015). Direct association of BOP1 at two TGA-binding sites in the *ATH1* promoter region was identified

by chromatin immunoprecipitation (ChIP) (Khan et al., 2015). Leaf material collected from *BOP1pro:BOP1-GR bop1 bop2* flowering plants was probed using an anti-GR antibody. Eight sites with TGA motifs spanning a 2178-base pair region upstream of the *ATH1* transcription start site were tested. Site IV (nt -2686 to -2577) with a 1.77-fold enrichment of BOP1 protein after DEX treatment was identified (Khan et al., 2015). Site VII (nt -1529 to -1416) was identified as a second potential binding site at lower confidence. Both of these sites contain an A-box motif, which is a strong binding site for TGA1 (Izawa et al., 1993).

To test if TGA1 and TGA4 were enriched at these sites, we first generated *tga1 tga4* transgenic plants harboring *TGA1pro:TGA1* and *TGA4pro:TGA4* translational fusions with *GFP* so we could use commercially available GFP antibody for the ChIP experiments. GFP fusions were expressed from the same native promoters as used for GUS lines (Figure 2.5). TGA1-GFP and TGA4-GFP fusion proteins localized in the nuclei of transgenic plants (Figure 2.9A). During a PTI response, various proteins are secreted into the apoplastic space. Tunicamycin inhibits N-glycosylation results in accumulation of secretory proteins in the endoplasmic reticulum. The loss-of-function *tga1 tga4* mutant lacks a proper response to endoplasmic reticulum stress hence susceptible to tunicamycin treatment (Wang and Fobert, 2013). The activity of fusion proteins was further monitored by complementation of *tga1 tga4* sensitivity to tunicamycin-induced endoplasmic reticulum stress (Wang and Fobert, 2013). Two *TGA1pro:TGA1-GFP tga1 tga4* and *TGA4pro:TGA4-GFP tga1 tga4* lines showing complementation of this defect were used for ChIP assays (Figure 2.9B). The ChIP assay showed significant enrichment of TGA1 at site IV and site VII in the *ATH1* promoter, whereas TGA4 interacted preferentially with site IV (Figure 2.9D and 2.9E). These results are consistent with the model that BOP1/2 interact with clade I TGAs for recruitment to the promoter of target genes.

2.5 Discussion

Arabidopsis BTB-ankyrin proteins BOP1/2 control plant architecture based on their activity at organ boundaries. These proteins are part of a larger family of NPR1-like proteins with roles in plant immunity. Despite these different functions, all proteins in this family have a similar domain structure and rely on interaction with bZIP TGA transcription factors for recruitment to DNA and activation of target genes. We show here that clade I TGA transcription factors, previously associated with plant defense, function broadly in BOP-dependent regulation of plant development. This work reveals a role for clade I TGAs at boundaries and hints at possible additional roles for this module in plant defense.

2.5.1 Clade I TGA bZIPs have dual functions in development and defense

Clade I TGA-like factors were originally identified biochemically in *Nicotiana tabacum* nuclear extracts, based on their ability to bind to an *activating sequence 1 (as-1)* element in the *Cauliflower Mosaic Virus* 35S promoter (Katagiri et al., 1989; Lam et al., 1989). The tandem TGACG motifs comprising this element drive expression of the 35S promoter in roots (Benfrey and Chua, 2011) and show responsiveness to signals like SA, jasmonic acid, the auxin-related herbicide 2,4-dichlorophenoxyacetic acid, and hydrogen peroxide associated with biotic and abiotic stress (Liu and Lam, 1994; Qin Xiao Feng et al., 1994; Xiang et al., 1996). Compatible with these findings, inactivation of *TGA1* and *TGA4* genes impairs pattern-triggered immunity (PTI) and to a lesser extent, NPR1-dependent SAR (Després et al., 2003; Kesarwani et al., 2007; Wang and Fobert, 2013; Sun et al., 2018). Clade I TGAs are required for the full induction of *SYSTEMIC ACQUIRED RESISTANCE1 (SARD1)* and *CALMODULIN-BINDING PROTEIN 60g (CBP60g)* genes needed for biosynthesis of SA and pipecolic acid during both types of immune response (Sun et al., 2018).

Clade I TGAs are also reported to have developmental roles. *tgal tga4* double mutants have altered root architecture under low nitrate conditions (Alvarez et al., 2014; Canales et al., 2017) and curved petioles (Shearer et al., 2012). Biochemical experiments show that TGA4 interacts with CONSTANS and binds to the *FLOWERING LOCUS T* promoter, suggesting formation of a complex that regulates flowering (Song et al., 2008).

Here, we characterize a role for clade I TGA1 and TGA4 transcription factors at organ boundaries. Both genes are strongly expressed in mature organ boundaries, including the base and petiole of leaves, axil of floral pedicels, and the base of floral organs. Despite this pattern, *tgal tga4* mutants have no obvious boundary-related defects, presumably due to redundancies. Transcripts for *TGA3* and *TGA9* are also upregulated in *BOP1*-overexpressing transgenic plants (Khan et al., 2015; Table 2.2) and may contribute. This suggestion is supported by studies in maize (*Zea mays*) showing that *LIGULELESS2* (most related to *AtTGA9*) contributes to establishment of the leaf blade-sheath boundary. Ligule and auricle structures formed at this junction are missing in *liguleless2* mutants, causing leaf blades to be more upright as they bend away from the stem (Walsh et al., 1998; Tian et al., 2011). *liguleless2* mutants are also delayed in flowering and have fewer, more upright, tassel branches (Walsh and Freeling, 1999).

To investigate a boundary function for clade I TGAs, we studied the impact of loss-of-function mutations on the phenotype of transgenic plants constitutively expressing *BOP1* or *BOP2*. Constitutive expression of *BOP1/2* inhibits stem elongation and flowering (Norberg et al., 2005; Khan et al., 2012a, 2015). Depletion of *TGA1* and *TGA4* activities in *35S:BOP2* plants restored internode elongation in the majority of F1 progeny suggesting that these factors contribute to BOP2 activity. To further examine the role of TGA1 and TGA4 in boundaries, we investigated

their interaction with PNY and PNF. Ectopic expression of boundary genes in the shoot apex of *pnf* and *pnf pnf* mutants impairs SAM maintenance, flowering, and stem elongation (Andrés et al., 2015; Khan et al., 2015). These defects are corrected by *bop1 bop2* (Khan et al., 2012b, 2015) and were also suppressed by *tga1*, *tga4*, or *tga1 tga4* mutations consistent with functions in the same genetic pathway. Similar onset of flowering in *tga1 pnf pnf* and *tga4 pnf pnf* compared to *tga1 tga4 pnf pnf* mutants suggests that TGA1 and TGA4 have interchangeable functions in the shoot apex. This contrasts with the stem, where *TGA1* inactivation had a greater impact on rescue of *pnf* and *pnf pnf* defects in silique clustering. Both *TGA1* and *TGA4* reporter genes showed an expanded pattern of expression in *pnf* and *pnf pnf/+* stems, suggesting that they are under the same regulatory control as other boundary genes. PNY directly represses *BOP1/2* in the shoot apex and stem to maintain organized patterns of cell division required for meristem activity, flowering, and stem elongation (Andrés et al., 2015; Bencivenga et al., 2016). Genome-wide analysis of PNY binding sites show interactions with the genomic DNA of numerous boundary genes, including *ATH1* and *KNAT6* functioning in the same genetic pathway as *BOP1/2*. The same study identifies *TGA1* and *TGA4* as having high-confidence binding sites for PNY, suggesting direct repression (Bencivenga et al., 2016).

2.5.2 Clade I TGAs are required for BOP-dependent activation of a boundary gene

BOP1/2 fulfill their function in part by promoting the expression of boundary genes, both directly and indirectly (Jun et al., 2010; Khan et al., 2012b; Khan, 2013; Khan et al., 2015). Our data provide evidence that clade I TGA factors are directly required for BOP-dependent activation of *ATH1*, encoding a BELL-type homeobox gene that functions in boundary patterning. Loss-of-function *ath1* mutations cause ectopic elongation of the region below the shoot apex in seedlings,

fusion of floral organs, and partial defects in floral organ abscission (Gómez-Mena and Sablowski, 2008). In young seedlings, *ATH1* transcripts were reduced similarly by *bop1 bop2* and *tga1 tga4* mutations. In inflorescence apices, *ATH1* transcripts were partially reduced in *tga1 tga4* mutants compared to *bop1 bop2*, suggesting increased redundancy during the reproductive stage. A 2-kb region of the *ATH1* promoter fused to GUS is responsive to BOP1 induction (Khan et al., 2015). In a *tga1 tga4* mutant background, BOP1 induction of this GUS reporter was markedly reduced, also suggesting that clade I TGAs are *ATH1* positive regulators. ChIP assays have demonstrated BOP1 binding to site VII within this 2-kb promoter and site IV, which is slightly upstream (Khan et al., 2015). These sites contain A-box motifs (TAC/GTA) whose two palindromic half sites have been shown biochemically to recruit TGA1 (Schindler et al., 1992; Izawa et al., 1993; Khan et al., 2015). Compatible with these data, we show that site IV is a binding site for TGA1 and TGA4 whereas site VII selectively binds TGA1. Nucleotides flanking the AC/GT core greatly influence the degree of binding (Schindler et al., 1992). These differences likely reflect unique binding requirements for TGA4, whose preference for flanking residues has not been determined biochemically. Collectively, these studies demonstrate a requirement for clade I TGAs in BOP1-dependent activation of *ATH1*. Genome-wide analysis of BOP1/2 binding sites is an important future direction in determining if this mechanism applies widely to the induction of genes important for boundary function.

2.5.3 Clade I TGAs interact differentially with BOP1/2 and NPR1

Clade I TGA recruitment of BOP1/2 to the promoter of *ATH1* is additionally supported by a physical interaction between BOPs and TGA1/4 detectable in yeast cells. An interaction is also observed in nuclei of *N. benthamiana* leaves using BiFC assays. Preferential interaction of BOP1/2

with native TGA1 and TGA4 contrasts with NPR1, whose interaction with clade I TGAs is redox-regulated. Interaction of TGA1 with NPR1 is stimulated *in planta* in the presence of SA, which changes the conformation of NPR1 and redox status of TGA1 and TGA4 (Després et al., 2003; Wu et al., 2012b). Mutations of specific Cys residues in TGA1 (C260/C266) and equivalent residues in TGA4 (C256/C262) bring about interaction with NPR1 in yeast (Després et al., 2003), suggesting that SA-induced reduction of an inhibitory disulphide bond promotes complex formation. This interaction also occurs in SA-treated leaves, but is not critical for SAR (Després et al., 2003; Kesarwani et al., 2007). Clade I TGAs function primarily in innate immunity based on transcriptome studies and backed by phenotypic data (Kesarwani et al., 2007; Shearer et al., 2012; Wang and Fobert, 2013; Sun et al., 2018). Interestingly, redox mimic mutations that promote NPR1 interaction with TGA1 and TGA4 (Després et al., 2003) reduce the interaction with BOP1 and BOP2 in yeast cells. Thus, selective recruitment of BTB-ankyrin proteins by clade I TGAs might depend on redox status. CC-type glutaredoxins (named ROXYs in Arabidopsis) are candidates for redox control. ROXYs interact physically and genetically with TGA bZIP factors in regulating both development and defense (Ndamukong et al., 2007; Zander et al., 2012; Gutsche et al., 2015; Uhrig et al., 2017). ROXY1 interacts with TGA8/PAN, whose redox-sensitive DNA-binding activity determines petal number in flowers (Li et al., 2009; Gutsche and Zachgo, 2016). Similarly, ROXY1 and ROXY2 interact with TGA9 and TGA10, whose functions are redundantly required for anther and pollen development (Murmu et al., 2010). ROXY1 weakly interacts with TGA1 and TGA4 in yeast, but the biological significance is untested (Li et al., 2009). Redox modification by nitrosylation increases the DNA-binding activity of TGA1 to target sites *in vitro* (Lindermayr et al., 2010). Alternatively, ROXY19/GRX480 interacts with TGA2 and TOPLESS-type transcriptional co-repressors in yeast, possibly functioning as a redox-regulated adaptor for

establishing TGA-dependent repression of target genes (Uhrig et al., 2017). During SAR, activated NPR1 enters the nucleus and interacts with TGA2, thereby masking its repression domain and providing an activation domain for induction of defense genes (Boyle et al., 2009). Clade I TGA recruitment of BOP1/2 may serve as a similar switch for activation of target genes involved in development or defense. Elevated basal levels of pathogenesis-related (PR) defense genes, including *PR1*, *PR2*, and *PR5*, in *tga1 tga4* mutants is consistent with at least a subset of genes under negative regulation (Lindermayr et al., 2010; Shearer et al., 2012). Future experiments will investigate this model.

Table 2.1 List of primers.

Sequence	Purpose	Forward sequence (5'-3')	Reverse sequence (5'-3')	Reference
<i>TGA1pro</i>	<i>TGA1pro:GUS</i> construct	GAAGGATCCATATCGACCAATTAATATAGG	GGACCATGGCCATGGCCATGTTTCCTCAACTGAAAAC	This study
<i>TGA4pro</i>	<i>TGA4pro:GUS</i> construct	GAAGGATCCGTGGGCTTTCAAAACTGATGG	GGACCATGGCCATATTTCTTCAACTAGCAAC	This study
<i>TGA1</i>	Genotyping <i>tga1-1</i> SALK_028212	TTCAAAACCTGGATTCATGGTTTCC	TAGGGAATCTCCGTGTCCCCTCTGG	This study
<i>TGA4</i>	Genotyping <i>tga4-1</i> SALK_127923	GCTCTGCTGAAGTTTTCCACATTCC	GAAGGTTTGAAGTTTACGAGCCTCT	This study
<i>TGA1pro:TGA1</i>	<i>TGA1pro:TGA1-GFP</i> fusion construct	CACCGCACGTGGCATTGCATGAGTCCAGTCC	CGTTGGTCCACGATGTCGAGT	This study
<i>TGA4pro</i>	<i>TGA4pro:TGA4-GFP</i> fusion construct	TGCAGCCCGGGGGATCGTGGGCTTTCAAAACTGATG	CTAGAACTAGTGGATCATTTCTTCAACTAGCAACAA	This study
<i>TGA4</i>	<i>TGA4pro:TGA4-GFP</i> fusion construct	ATGAATACAACCTCGACACA	TTCTCCTTTACTCATCGTTGGTTCACGTTGCCT	This study
<i>GFP</i>	<i>TGA4pro:TGA4-GFP</i> fusion construct	ATGAGTAAAGGAGAAGAAGCTT	TGGCGGCCGCTCTAGTTAGTGGTGGTGGTGGTG	This study
<i>TGA1</i>	RT-qPCR	ATGAATTTCGACATCGACACATTTTG	ATCCTCTGACACGTTGTTGTCTAG	This study
<i>TGA4</i>	RT-qPCR	ATGAATACAACCTCGACACATTTG	AAGTATCCTCTGACAAGCTGTCTGG	This study
<i>ATH1</i>	RT-qPCR	ATACTCGCTCGATTATTCATCTCGA	ATCGATCATCCACCATTGAAGAAG	Khan et al., 2015
<i>GAPC</i>	RT-qPCR	TCAGACTCGAGAAAGCTGCTA	GATCAAGTCGACCACACGG	Khan et al., 2012b
<i>TUB4</i>	RT-qPCR	GGTCAATACGTCGGCGATTCC	TCTGACCGAACGGACCAGAT	This study

<i>ELF4</i>	RT-qPCR	AAACTCAATGAAGTACTTGAGGGAC	TCTCAAAACCATAAGCATAAAATACCC	Khan et al., 2015
site IV <i>ATH1pro</i>	ChIP-qPCR	TATGTTTAGGTGGATGGCT	TCTGAAGCACTTGAGGGTC	Khan et al., 2015
site VII <i>ATH1pro</i>	ChIP-qPCR	AGGAACAATCAAAGAAAAGG	GTATTGTGCCATCAAATCTTAT	Khan et al., 2015
<i>UBQ5</i> promoter	ChIP-qPCR	GACGCTTCATCTCGTCC	GTAAACGTAGGTGAGTCCA	Boyle et al., 2009
<i>TGA1</i>	Gateway donor vector construct	ATGAATTCGACATCGACACATTTTG	CGTTGGTCCACGATGTCGAGT	This study
<i>TGA4</i>	Gateway donor vector construct	ATGAATACAACCTCGACACATTTTG	CGTTGGTTCACGTTGCCTAGC	This study
<i>BOP1</i>	Gateway donor vector construct	ATGAGCAATACTTTCGAAGAATC	GAAATGGTGGTGGTGGTGATG	This study
<i>BOP2</i>	Gateway donor vector construct	ATGAGCAATCTTGAAGAATCTTTG	GAAGTGATGTTGATGATGGTG	This study
<i>PAN</i>	Gateway donor vector construct	ATGCAGAGCAGCTTCAAAACCGTTC	GTCTCTAGGTCTGGCTAACCA	This study
<i>mTGA1</i>	Gateway donor vector construct	CACCATGAATTCGACATCGACACAT	CGTTGGTCCACGATGTCGAGT	This study
<i>mTGA4</i>	Gateway donor vector construct	CACCATGAATACAACCTCGACACAT	CGTTGGTTCACGTTGCCTAGC	This study

Table 2.2 List of differentially expressed *TGA* genes

Gene	Accession number	Fold change (<i>BOP1-oe</i> vs WT)
<i>TGA4</i>	At5g10030	3.10
<i>TGA3</i>	At1g22070	1.49
<i>TGA9</i>	At1g08320	1.43

Figure 2.1 Clade I TGA transcript analysis in transgenic plants overexpressing *BOP1* and phenotype of *tga1 tga4* double mutants.

(A) *TGA1* and *TGA4* transcripts in transgenic plants overexpressing *BOP1* as determined by RT-qPCR analysis on internodes of wild-type (WT) and *BOP1-oe* plants. Data represent means $\pm$ SD of three biological replicates. Asterisks, significantly different from WT (Student's *t* test, $p < 0.01$). (B-G), Inactivation of *TGA1* causes petiole curvature. Plants were grown under long days. B, Wild type. C, *bop1 bop2* mutant; helical growth of leaves. D, *tga1* mutant; helical growth of leaves. E, *tga4* mutant; similar to wild type. F, *tga1 tga4* double mutant; strong helical growth of leaves. G, *tga1 tga4 bop1 bop2* quadruple mutant; strong helical growth of leaves. Scale bar, 1 cm.

Figure 2.2 Requirement of TGA1 and TGA4 for BOP-dependent regulation of inflorescence architecture.

Plants homozygous for a *35S:BOP2* transgene were crossed to wild-type Col plants or homozygous *tga1*, *tga4*, or *tga1 tga4* mutants. Representative F1 progenies are shown. Number of plants with restored stem elongation (>12 cm tall) in population is given at the top right of panels. (A) *35S:BOP2*/+ Col/+ control plants; dwarf. (B) *35S:BOP2*/+ *tga1*/+ plant; similar to control plant. (C) *35S:BOP2*/+ *tga4*/+; similar to control plant. (D) *35S:BOP2*/+ *tga1*/+ *tga4*/+ plant; stem elongation is restored. Scale bars, 2cm.

Figure 2.3 Inactivation of BOP1/2, TGA1, and TGA4 corrects *pny pnf* meristem arrest.

Representative images are shown of 25-day-old plants grown under short days. Number of plants showing a meristem arrest are indicated at top right of panels. (A) Wild type. (B) *pny pnf* mutant showing a SAM arrest (arrow, no new leaves). (C) *bop1 bop2 pny pnf* mutant; no meristem arrest. (D) *tga1 pny pnf* mutant; no meristem arrest. (E) *tga4 pny pnf* mutant; no meristem arrest. (F) *tga1 tga4 pny pnf* mutant; no meristem arrest. Scale bars, 5 mm.

Figure 2.4 Loss of TGA1 and/or TGA4 corrects *pny* and *pny pnf* defects in flowering and inflorescence architecture.

Representative images shown for 6-week-old plants grown under continuous light. (A) Wild-type inflorescence apex. (B) *pny* mutant; clustered siliques (arrows). (C) *tga1 pny* mutant; partial rescue of clustering (arrow). (D) *tga4 pny* mutant; little or no rescue of clustering (arrows). (E) *tga1 tga4 pny* mutant; no clusters. (F) Wild type. (G) *pny pnf* mutant; non-flowering. (H) *tga1 pny pnf* mutant; flowering is restored, partial rescue of clustered siliques (arrow). (I) *tga4 pny pnf* mutant; flowering is restored, siliques are clustered (arrow). (J) *tga1 tga4 pny pnf* mutant; similar to wild type. Scale bars, 1.5 cm.

Figure 2.5 *TGA1* and *TGA4* expression is enriched at boundaries in the inflorescence in a pattern that overlaps with *BOP1* and *BOP2*.

Arrows denote enriched areas of expression. (A-G) *TGA1pro:GUS*. (A) 10-day-old seedling. (B) Rosette leaf, petiole. (C) Inflorescence apex. (D) Longitudinal section of young flower. (E) Mature fruit, abscission zones. (F) Inflorescence stem. (G) Stem cross-section. (H-N) *TGA4pro:GUS*. (H) 10-day-old seedling. (I) Rosette leaf, petiole. (J) Inflorescence apex. (K) Longitudinal section of young flower. (L) Mature fruit; abscission zones. (M) Inflorescence stem. (N) Stem cross-section. (O-U) *BOP1pro:GUS*. (O) 10-day-old seedling. (P) Rosette leaf, petiole. (Q) Inflorescence apex. (R) Longitudinal section of young flower. (S) Mature fruit; abscission zones. (T) Inflorescence stem. (U) Stem cross-section. (V-Z'') *BOP2pro:GUS*. (V) 10-day-old seedling. (W) Rosette leaf, petiole. (X) Inflorescence apex. (Y) Longitudinal section of young flower. (Z) Mature fruit; abscission zones. (Z') Inflorescence stem. (Z'') Stem cross-section. Xylem (X) and Phloem (Ph). Scale bars, 1 mm except DGKNRUYZ'', 50 μ m.

Figure 2.6 Expression of *TGA1* and *TGA4* in wild-type and mutant inflorescences.

(A-C) *TGA1pro:GUS*. (A) Wild-type; expression at the base of pedicels, flowers, fruits, and stem vasculature (close-up). (B) *pny* mutant; strong upregulation in the stem (arrows) concentrated at nodes (close-up). (C) *pny pnf/+* mutant; strong upregulation in the stem (arrow) concentrated at nodes (close-up). (D-F) *TGA4pro:GUS*. (D) Wild-type, expression pattern similar to (A) *TGA1pro:GUS*. (E) *pny* mutant; strong upregulation in the stem (arrows) concentrated at nodes (close-up). (F) *pny pnf/+* mutant; strong upregulation in the stem (arrows) concentrated at nodes (close-up). Scale bars, 0.5 mm.

Figure 2.7 Clade I TGAs are required for BOP1 induction of *ATH1* expression.

(A-B) *ATH1* transcript levels were measured in wild-type (WT) and mutants using RT-qPCR. (A) 10-day-old seedlings. (B) Inflorescence apices. Data are means $\pm$ standard error from three independent experiments with the same letter indicating no significant difference at $p \leq 0.05$ (ANOVA with Duncan's post-hoc test). (C-G) Induction of 2-kb *ATH1pro:GUS* by *D35S:BOP1-GR* was disrupted by inactivation of *TGA1* and *TGA4*. (C-D) 2-kb *ATH1pro:GUS D35S:BOP1-GR* in wild-type Col-0 background. Images are representative. (C) Inflorescence apex from mock treated plant. *ATH1* expression was mainly at the base of floral organs. (D) Inflorescence apex from 10 μ M DEX-treated plant. *ATH1* expression was induced in flowers, pedicels, and pedicel axils. (E-G) 2-kb *ATH1pro:GUS D35S:BOP1-GR* in *tga1 tga4* mutant. (E) Inflorescence apex from mock treated plant. *ATH1* expression pattern is similar to mock Col-0 plants. (F-G) Inflorescence apex from 10 μ M DEX-treated plants. (F) Intermediate induction (8.74% of plants) and (G) weak induction (91.26% of plants) compared to DEX-treated Col-0 plants (n=87 for WT; n=183 for *tga1 tga4*). Scale bars, 1 mm. H, Measurement of plant height for DEX-induced plants (n=18 for WT; n>36 for *tga1 tga4*). Data are means $\pm$ standard deviation. Asterisk, significant difference between DEX-treated WT and *tga1 tga4* plants (Student's *t* test, $p < 0.01$).

Figure 2.8 Biochemical evidence of BOP1 and BOP2 interaction with TGA1 and TGA4.

(A) Pair-wise yeast two-hybrid assays showing BOP1 and BOP2 interaction with wild-type and cysteine mutant forms of TGA1 and TGA4. BOP1 and BOP2 baits were fused to the DNA-binding domain of yeast GAL4. TGA preys were fused to the transcriptional activation domain of yeast GAL4. Plasmid constructs were co-transformed into yeast AH109 strain before serial dilutions (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}) were plated onto SD/-Trp/-Leu medium with or without histidine + 8 mM 3-AT. Growth on SD/-Trp/-Leu/-His/+3-AT above background confirms a protein-protein interaction. Dilutions were spotted in replicate onto SD/-Trp/-Leu/-His/+3-AT medium supplemented with 20 mg l⁻¹ X- α -gal for assessment of α -galactosidase reporter activity. Photos on SD/-Trp/-Leu were taken two days after plating. Photos on SD/-Trp/-Leu/-His/+3-AT with or without X- α -gal were taken after three days. For all assays, interaction with PAN was used as a positive control and an empty prey vector was used as a negative control. (B) BOP1 and BOP2 interaction with TGA1 and TGA4 in nuclei of transiently-transformed *N. benthamiana* leaves. BiFC was used to examine BOP1/2 interactions with TGA1 and TGA4 *in planta*. The N-terminus of YFP (nYFP) was fused in-frame at the N-terminus BOP1 or BOP2. The C-terminus of YFP (cYFP) was fused in-frame at the N-terminus of PAN, TGA1, or TGA4. Pairs of constructs were transiently expressed in *N. benthamiana* leaves. Reconstitution of YFP fluorescence was examined by confocal microscopy four days after infiltration. Yellow fluorescence in the nucleus was detected for BOP1 and BOP2 paired with PAN, TGA1, and TGA4 (arrows). Red fluorescence, chlorophyll *a* autofluorescence. Blue fluorescence, DAPI-stained nuclei. Scale bars, 20 μ m.

Figure 2.9 TGA1 and TGA4 bind to the same *cis* elements as BOP1/2 in the *ATH1* promoter based on ChIP assays.

(A) Confocal microscope images of *TGA1pro:TGA1-GFP tga1 tga4* and *TGA4pro:TGA4-GFP tga1 tga4* seedlings showing nuclear localization of TGA1-GFP and TGA4-GFP. (B) *TGA1pro:TGA1-GFP tga1 tga4* and *TGA4pro:TGA4-GFP tga1 tga4* lines for ChIP are complemented for tunicamycin (TM) sensitivity. Five-day-old seedlings (n=5 per treatment group, 5-6 replicates) were treated with 0.8 $\mu\text{g ml}^{-1}$ TM or water for 6 hours. The fresh weight of TM- versus mock-treated seedlings after five days of recovery on TM-free media is shown. Data are means $\pm$ standard deviation. Asterisk, significant difference compared to WT (Student's *t* test, $p < 0.05$). (C) Map of the *ATH1* promoter and 5' untranslated region. Closed arrowhead marks the transcription start site. Predicted consensus A-box binding sites for TGA bZIP factors are marked with asterisks. ChIP-qPCR amplified fragments are underlined. (D) and (E) ChIP-qPCR assay showing that TGA1 and TGA4 bind to the *ATH1* promoter. Apices of 6-week-old plants were harvested for ChIP using anti GFP-antibody. qPCR was performed to calculate the abundance of specific regions of genomic DNA. Each ChIP-qPCR histogram indicates the mean $\pm$ standard error of three biological replicates, except (D) where only two biological replicates were quantified for TGA4. Enrichment values were normalized to input DNA. For each biological replicate, three technical qPCR reactions were performed. Asterisks, significant difference compared to control (Student's *t* test *, $p < 0.05$; **, $p < 0.01$).

Supplemental Figure S2.1 Quantitative analyses of *pny* and *pny pnf* phenotypic rescue by *tgal* and/or *tga4*.

(A-B) Quantitative analyses of *pny* phenotypic rescue were conducted on 8-week-old plants grown under long days. Twenty-four plants per genotype were scored. (A) Distribution of internode lengths between successive siliques. Internodes between the 1st and 11th siliques (counting acropetally) were measured. Distribution of internode lengths in the *tgal tga4 pny pnf* quadruple mutant was also similar to wild-type (data not collected). (B) Average number of basal paraclades in wild-type and mutants. Loss-of-function *tgal*, *tga4*, or *tgal tga4* mutations restore apical dominance in *pny* mutants. Data are means $\pm$ standard error. (C) Quantitative of flowering time in wild-type and mutants. Rosette (black) and cauline (white) leaves were marked and counted daily until plants were mature. Data are mean $\pm$ standard error ($n \geq 72$). The flowering time of *tgal tga4 pny pnf* mutants was similar to wild-type.

Supplemental Figure S2.2 *TGA1pro:GUS* and *TGA4pro:GUS* expression in wild-type and *pny pnf* shoot apices

(A-C) *TGA1pro:GUS*. (A) 10-day-old wild-type seedling. Longitudinal section showing expression in young leaves and hypocotyl. (B) 10-day-old *pny pnf* seedling. Longitudinal section showing intensified expression in the hypocotyl. (C) Inflorescence apex showing expression in the stem vasculature, particularly at the base of floral pedicels. (D-F), *TGA4pro:GUS*. (D), 10-day-old wild-type seedling. Longitudinal section showing expression in young leaves and hypocotyl. (E) 10-day-old *pny pnf* seedling. Longitudinal section showing intensified expression in the hypocotyl and base of leaves. (F) Inflorescence apex showing expression in the stem vasculature. Scale bars, 50 μ m.

CHAPTER 3

Arabidopsis BLADE-ON-PETIOLE1 and 2 interact with clade I TGA and WRKY transcription factors to promote plant defense

This chapter is presented essentially as submitted to Plant Cell (in revision)

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3.1 Abstract

Arabidopsis BLADE-ON-PETIOLE 1 (BOP1) and BOP2 form a subclade of BTB-ankyrin proteins within the NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) family of defense regulators. BOP1/2 interact with clade I TGACG-binding factors TGA1 and TGA4 to regulate development at organ boundaries. Growth is restricted at boundaries such that plants overexpressing *BOP1* or *BOP2* are dwarf. Here, *BOP1*-overexpressing plants were characterized to reveal increased pathogen defenses. Pathology assays showed that pattern triggered immunity (PTI) responses were enhanced in *BOP1*-overexpressing plants but decreased in *bop1 bop2* and *tga1 tga4* mutants compared to wild type. Analyses of transcriptome data showed a significant enrichment of defense genes clustered with PTI where TGA1/4 play a role. The transcriptomes of PTI-elicitor flagellin22-treated *bop1 bop2* and *tga1 tga4* mutants exhibited similar profiles. Mechanistically, we showed that BOP1 binds to TGACG-containing regions in the promoters of TGA1/4-regulated *SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1* (*SARD1*) and *CALMODULIN BINDING PROTEIN 60g* (*CBP60g*) but only in response to flagellin22. The promoters of BOP1-upregulated genes, including *SARD1* and *CBP60g*, contained abundant W-box motifs, suggesting WRKY transcription factors as binding partners for BOP1/2 and clade I TGAs. Further biochemical evidence proved a complex can be formed among WRKY transcription factors with both BOP1/2 and clade I TGAs. These data show a defense role for BOPs using functional partners similar to NPR1.

3.2 Introduction

Plants integrate multiple environmental signals to fine-tune the opposing needs of growth and defense. Upon pathogen perception, defense responses are activated at the expense of growth (Huot et al., 2014). Failure to tightly control this activation can lead to autoimmunity, which is detrimental to plant growth (van Wersch et al., 2016). Plants can use both innate (localized) and induced (systemic) forms of immunity to resist pathogens (Jones and Dangl, 2006; Pieterse et al., 2012; Zhou and Zhang, 2020). Pattern-triggered immunity (PTI) is the first layer defense, which relies on the local perception of molecules with conserved motifs, named pathogen-associated molecular patterns (PAMPs), by receptors on the cell surface (Macho and Zipfel, 2014; Bigeard et al., 2015). One of the most well-documented PTI responses in *Arabidopsis thaliana* (Arabidopsis) is initiated by perception of a conserved 22-amino-acid fragment of bacterial flagellin named flg22 by the membrane receptor FLAGELLIN SENSITIVE2 (FLS2) (Gómez-Gómez and Boller, 2000; Macho and Zipfel, 2014). Following the perception of flg22, a rapid and transient burst of reactive oxygen species (ROS) is produced, ultimately leading to the expression of genes that fortify the cell perimeter with barriers to infection including release of antimicrobial compounds, deposition of callose, lignin polymerization, and cuticle thickening (Jones and Dangl, 2006; Pieterse et al., 2012; Zhou and Zhang, 2020).

Some pathogens have evolved specialized macromolecules called effectors that suppress PTI responses and promote disease. To detect invasion by these specialized pathogens, resistant plants employ intracellular receptors that recognize effector proteins from the pathogens, leading to a more robust local response known as effector-triggered immunity (ETI). This second layer of immunity is often associated with programmed cell death at the infection site, known as

hypersensitive response. These localized responses function not only to prevent pathogen invasion, but act as a priming mechanism, enabling the plant to have heightened resistance against future attacks (Henry et al., 2013; Bigeard et al., 2015).

Phytohormones play a major role in orchestrating plant defense. Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are the main hormones associated with plant immunity (Bigeard et al., 2015). All three hormones are produced at the site of attack leading to specific outcomes tailored to the pathogen. SA signaling is the dominant response to biotrophic or hemibiotrophic pathogens like *P. syringae* whereas JA/ET signaling is dominant against necrotrophs (Glazebrook, 2005; Pieterse et al., 2012). SA-dependent signaling can lead to a longer-lasting systemic response known as systemic acquired resistance (SAR) of which NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) is the master regulator (Fu and Dong, 2013; Zhou and Zhang, 2020).

NPR1 belongs to a plant-specific family of transcription co-factors characterized by a BTB/POZ (Broad-complex, Tramtrack, and Bric-a-brac/POX virus and zinc finger) domain and ankyrin repeats. Both of these domains are surfaces for protein-protein interactions that occur in the cytoplasm or nucleus (Khan et al., 2014; Backer et al., 2019). BTB-ankyrin proteins in mosses and higher plants including *Arabidopsis* encompass two phylogenetic subclades (Wang et al., 2015; Saleh et al., 2011). *Arabidopsis* NPR1 and its three close homologs (NPR2, NPR3, and NPR4) have related roles in SA-mediated plant defense (Cao et al., 1997; Canet et al., 2010; Fu et al., 2012; Ding et al., 2018; Backer et al., 2019; Zavaliev et al., 2020) whereas another subclade containing BLADE-ON-PETIOLE 1 (BOP1) and BOP2 regulate plant development (Chan et al., 2004; Ha et al., 2003; Hepworth et al., 2005; Norberg et al., 2005). A role for NPR1 in plant

defense was first identified based on loss-of-function mutations that eliminated SAR (Cao Hui et al., 1994; Cao et al., 1997). Following local infection, systemically elevated levels of SA lead to conversion of NPR1 to a monomeric state, allowing its transportation from the cytoplasm to the nucleus (Mou et al., 2003; Tada et al., 2008). In the nucleus, NPR1 interacts with various TGA bZIP transcription factors, thereby activating the expression of defense genes such as *PATHOGENESIS RELATED GENES 1 (PR1)* (Zhang et al., 1999; Despres et al., 2000; Zhou et al., 2000). Multiple TGAs are involved in NPR1-dependent SAR responses. Clade II TGAs interact with NPR1 and play a major role in SAR, since knockout mutants of TGA2, TGA5, and TGA6 show defects in SA-dependent gene expression and disease resistance similar to *npr1* mutants (Zhang et al., 2003). NPR1 also interacts with clade III TGA3 (Choi et al., 2010; Saleh et al., 2015; Chen et al., 2019a) and with redox-modified forms of clade I TGA1 and TGA4 to regulate SA-dependent defenses (Després et al., 2003; Sun et al., 2018).

WRKY transcription factors are another class of activators and repressors that function prominently in plant defense (Eulgem, 2005; Pandey and Somssich, 2009; Rushton et al., 2010). Numerous WRKY genes are quickly up-regulated in response to PAMP exposure (Dong et al., 2003; Zipfel et al., 2004). A significant number of co-regulated genes have W-box elements within their promoter regions suggesting they are WRKY-regulated (Maleck et al., 2000; Birkenbihl et al., 2017). WRKY6 was one of the first examples of a positive regulator in plant defense. Two defense marker genes *PR1* and *FLG22-INDUCED RECEPTOR-LIKE KINASE 1/SENESCENCE-INDUCED RECEPTOR-LIKE KINASE* are among its targets during an immune response (Robatzek and Somssich, 2002, 2001). In contrast, WRKY70 represses pathogen-inducible genes including *SYSTEMIC ACQUIRED RESISTANCE DEFICIENT1 (SARD1)* in uninfected plants (Jiang et al., 2016; Zhou et al., 2018). However, relatively little is known about how WRKYs

interact with TGA factors or NPR1 in regulating immunity (Withers and Dong, 2016; Backer et al., 2019).

BOP1 and BOP2 have a divergent C-terminus compared to NPR1 and function prominently in plant development based on loss- and gain-of-function mutant phenotypes (Norberg et al., 2005; Khan et al., 2014). In aerial tissues, *BOP1/2* expression is enriched at organ boundaries where growth is restricted in controlling apical meristem activity, organ separation, and the shape and complexity of lateral organs (Hepworth et al., 2005; Khan et al., 2014; Hepworth and Pautot, 2015). Boundaries also influence plant architecture as sites for the production of axillary meristems and abscission (Hepworth and Pautot, 2015). Major boundary-related defects are observed in *bop1 bop2* mutants including organ fusions, elongated leafy petioles, abnormal floral patterning, and loss of floral organ abscission (Hepworth et al., 2005; Norberg et al., 2005). *BOP1/2* are also expressed in the hypocotyl and roots where they control secondary growth (Liebsch et al., 2014; Woerlen et al., 2017). In legumes, root elongation and lateral root conversion to symbiotic nodules also relies on *BOP* function (Couzigou et al., 2012; Magne et al., 2018; Shen et al., 2019). Conversely, gain-of-function *BOP* mutants have a dwarf phenotype and deposit excess lignin in their stems (Norberg et al., 2005; Khan et al., 2012a). In the cotton plant, *Gossypium hirsutum*, GhBOP1 can be spatially induced outside of organ boundaries to defend against a fungal pathogen, *Verticillium dahliae*, through interaction with GhTGA3, and the deposition of lignin (Zhang et al., 2019).

BOPs exert their developmental functions in part by interacting with bZIP TGA factors. We showed previously that BOP1/2 can interact with clade V TGA8/PAN to regulate floral patterning and more broadly with clade I TGA1 and TGA4 to regulate meristem function and

inflorescence patterning (Hepworth et al., 2005; Wang et al., 2019). Contrasting with NPR1, BOP1/2 interact preferentially with native forms of TGA1 and TGA4 in targeting the activation of *ARABIDOPSIS THALIANA HOMEBOX GENE 1 (ATH1)*, which patterns the basal boundaries of shoot organs (Després et al., 2003; Gómez-Mena and Sablowski, 2008; Wang et al., 2019). Interestingly, previous characterization of *tga1 tga4* mutants identified deficiencies in innate immunity including pathogen susceptibility, reduced apoplastic defenses, and impaired SA biosynthesis (Shearer et al., 2012; Wang and Fobert, 2013; Sun et al., 2018). Transcriptome analysis comparing *npr1* and *tga1 tga4* mutants showed only a small subset of genes co-regulated by NPR1 and clade I TGAs (Shearer et al., 2012). Among these target genes, *SARD1* and *CALMODULIN-BINDING PROTEIN 60g (CBP60g)* encode two related plant-specific transcription factors required for pathogen-induced SA and pipecolic acid biosynthesis (Zhang et al., 2010; Wang et al., 2011; Sun et al., 2018). Chromatin immunoprecipitation (ChIP) assays demonstrated direct binding of TGA1 to the *SARD1* promoter in a region that contains TGACG motifs (Sun et al., 2018). We therefore investigated a potential role for Arabidopsis BOP1 and BOP2 in plant defense pathways where clade I TGAs are active.

In this study, transcriptome analysis of *BOP1*-overexpressing (*BOP1-oe*) plants was used to uncover a defense role in PTI. Global analysis of gene expression depicted an immune response for *BOP1-oe* plants similar to wild-type plants treated with flg22. Further testing showed that *bop1 bop2* and *tga1 tga4* mutants were deficient in PTI responses whereas *BOP1-oe* response was heightened. A real-time quantitative PCR analysis showed that flg22-induction of several key PTI genes, including *SARD1* and *CBP60g*, was impaired in *bop1 bop2* plants. RNA-sequencing of *bop1 bop2* and *tga1 tga4* flg22-treated seedlings further demonstrated overlapping impairment of transcriptional response. ChIP assays showed that BOP1 recruitment to *SARD1* and *CBP60g*

promoters was flg22-dependent. WRKY transcription factors were identified as selective binding partners of BOP1/2 and TGA1/4 depicting a mechanism similar to NPR1.

3.3 Materials and methods

3.3.1 Plant material and growth conditions

Arabidopsis thaliana plants of the Columbia-0 (Col-0) ecotype were used in this study. The *bop1-6D* gain-of-function mutant (*BOP1-oe*) contains four viral 35S promoter copies in the promotor region of *BOP1* that drive high expression (Norberg et al., 2005). Loss-of-function mutants *bop1-3 bop2-1* (Hepworth et al., 2005), *tga1-1 tga1-4* (Kesarwani et al., 2007), *bop1-3 bop2-1 tga1-1 tga4-1* (Wang et al., 2019) and *fls2-17* (Zipfel et al., 2004) were as previously described. Transgenic *BOP1p:BOP1-GR bop1 bop2* plants used for ChIP have also been described (Khan et al., 2015).

For general propagation of plants, surface-sterilized seeds were sown on minimal media agar plates (Haughn and Somerville, 1986). Seven to nine-day-old seedlings were transplanted to steam-sterilized soil (Promix BX Black, Premier Horticulture) supplemented with 0.1% (w/v) of 20-20-20 plant fertilizer (Plant-Prod Inc., Brampton, ON, Canada). For seedling-based assays, surface-sterilized seeds were germinated on agar plates containing Murashige and Skoog (MS) modified basal medium with Gamborg vitamins (M404, PhytoTechnology Laboratories, Lenexa, KS, USA) and 1% sucrose (w/v).

Plants were grown in chambers at 21-22°C under short days (8 h light/16 h dark), long days (16 h light/8 h dark), or neutral days (12 h light/12 h dark) or on racks under continuous light (24 h light/0 h dark) as indicated (light intensity $\sim 115 \mu\text{mol m}^{-2} \text{sec}^{-1}$).

3.3.2 Microarray analysis

Microarray analysis was performed previously as described (Khan et al., 2015). Briefly, total RNA was harvested from the first expanded internodes of wild-type and *BOP1-oe* flowering plants grown under continuous light. RNA samples were prepared for four biological replicates per genotype using a 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 previously as described (Xiang et al., 2011; Khan et al., 2015). Data were analyzed using BioConductor tools implemented in R (Gentleman et al., 2004; Khan et al., 2015) resulting in a list of DEGs in *BOP1-oe* plants using log₂-fold increase or decrease ≥ 0.5 as the cut-off (Supplemental Data Set 1).

3.3.3 GO and GSEA analysis

Gene Ontology (GO) enrichment analysis was used to sort differentially expressed genes identified by microarray and RNA sequencing data into functional categories (Ashburner et al., 2000). GO analysis was performed at TAIR using PANTHER against the TAIR11 genome annotation release 2019-07-13 (Mi et al., 2013). The data were analyzed statistically with default parameters (Fisher's Exact Test with Bonferroni Correction) using a p-value cut-off of 0.05 as significant. GSEA was carried out using GSEA 4.0.0 (a joint project of UC San Diego and Broad Institute) as described in Supplemental Figure 3.1.

3.3.4 RT-qPCR

For microarray validation, total RNA was isolated from 10-day-old seedlings (10-15 seedlings per replicate) or the internodes of flowering plants (5-6 plants per replicate) using a Plant Total RNA

Mini Kit according to the manufacturer's instructions (Geneaid Biotech, Froggabio, Toronto, ON). The extracted total RNAs were treated with DNase I to remove residual genomic DNA as instructed by the manufacturer (Froggabio, Toronto, ON). The cDNA was synthesized using 1 µg of total RNA with Superscript III 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. RT-qPCR reactions were performed in triplicate using Invitrogen Power SYBR[®] Green PCR Master Mix (Thermo Fisher Scientific, Markham, ON) and run on an Applied Biosystems StepOnePlus thermocycler (Thermo Fisher Scientific, Markham, ON). Gene-specific primers were as listed in Supplemental Table 1. Differences in gene expression, expressed as a fold-change relative to the wild type, were calculated as described (Pfaffl, 2001). Values were normalized to *GLYCERALDE-3-PHOSPHATE DEHYDROGENASE C (GAPC)* and *ACTIN1 (ACT1)* (Khan et al., 2015; Sun et al., 2018).

For quantification of flg22-induced gene expression, sterilized seeds were sown on MS agar plates with 1% sucrose under continuous light at room temperature. Six-day-old seedlings were transferred to 24-well culture plates (one seedling per well) containing 500 µl of liquid MS media per well. After four days, seedlings were treated with 1 µM flg22 peptide for 0 hours (control) and 4 hours (induction). Samples were collected for three biological replicates (10-12 seedlings each). RNA was extracted and analyzed by RT-qPCR as above. Values were normalized to *ACT1* and *UBIQUITIN10 (UBQ10)* (Zhang et al., 2014; Sun et al., 2018).

3.3.5 RNA sequencing

Arabidopsis seedlings were germinated on MS agar plates supplemented with 1% sucrose under continuous light. Four days after germination, seedlings were transferred to 24-well tissue culture

plates containing 500 μ l of liquid MS media containing 1% sucrose per well. After ten days, this solution was replaced with fresh liquid media with or without 1 μ M flg22 peptide (P6622, PhytoTechnology Laboratories, Lenexa, KS, USA) for 4 hours. After flg22 treatment, seedlings were collected and blot dried, then flash-frozen in liquid nitrogen. Total RNAs were extracted using RNeasy Plant Mini kit (QIAGEN, China) followed by DNase I (QIAGEN, China) treatment. RNA integrity and concentration were assessed using a Bioanalyzer 2100 (Agilent Technologies, CA, USA). Sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (E7530L, NEB, USA) following the manufacturer's instructions. Pair-end sequencing was performed on Illumina HiSeq X Ten. Raw reads can be accessed at SRA under accession GSE157422. Script in R was used to trim and clean raw reads (library construction, sequencing, and raw reads processing were commercially contracted to ANNOROAD, Beijing, China). Reads with more than 5% ambiguous bases (N) were also removed. Clean reads were aligned to *Arabidopsis thaliana* genome (TAIR10) using FeatureCounts (Ver. 1.6.4). Aligned reads were then mapped using DESeq2 (Ver. 2.11.40.6) implemented in Galaxy-Europe (<https://usegalaxy.eu>). Two biological replicates were used to profile the transcriptome. When calculating differentially expressed genes, a gene was retained only if it had a log₂ (fold-change) ≥ 1 or ≤ -1 with an p-adj <0.05. Gene-based clustering and PCA analysis was performed in DESeq2 using default parameters.

3.3.6 Chromatin immunoprecipitation (ChIP) assays

A steroid-inducible form of BOP1 fused to the steroid-binding domain of the rat glucocorticoid receptor was used for ChIP assays (Khan et al., 2015). *BOP1pro:BOP1-GR bop1 bop2* seedlings were germinated under continuous light on MS agar plates with 1% sucrose $\pm$ 5 μ M

dexamethasone (DEX) (D4902, Sigma-Aldrich, St. Louis, MO). On day 7, seedlings were transferred to 24-well cell culture plates (one seedling per well) containing 800 μ l of liquid MS media $\pm$ 5 μ M DEX per well. On day 14, the MS media was refreshed and DEX-treated seedlings were separated into two groups: one group was continuously treated with MS + 5 μ M DEX and the other group was treated with MS + 5 μ M DEX + 1 μ M flg22 (P6622, PhytoTechnology Laboratories, Lenexa, KS). After 24 hours, all seedlings were harvested into liquid nitrogen. ChIP was performed as previously described (Gendrel et al., 2005)(Gendrel et al., 2005) using an anti-GR antibody (Ab3580, Abcam, Cambridge, UK). Quantification of immunoprecipitated DNA was performed by RT-qPCR using primers adopted from Sun et al. (2018) as indicated (Supplemental Table 3). Fold-enrichment was calculated using the input percentage of GR-immunoprecipitated samples relative to no-antibody control samples. The data shown are the average $\pm$ SE of three biological replicates. Three technical replicates were performed per biological replicate.

3.3.7 Pathogen assays

Plants were assayed for resistance against *P. syringae* pathovar (pv) *tomato* DC3000 (*Pst* DC3000) or a *hrcC* mutant of *Pst* (*Pst HrcC*⁻) as described (Shearer et al., 2012). Briefly, bacteria were grown at 28°C on King's *B* medium containing Rifampicin (100 mg l⁻¹). Overnight cultures were resuspended in infiltration medium (10 mM MgCl₂). Leaves of four-week-old plants grown under short days (8 h light/16 h dark at 22°C) were syringe-infiltrated with bacteria at 10⁵ colony-forming units (cfu) ml⁻¹. Samples were collected at 0-4 days post-inoculation. Each replicate consisted of 4 leaf discs (1 cm²) from the infected leaves of four different plants. Three replicates were collected per genotype. Bacterial titers were determined by plating serial dilutions of ground samples on Lysogeny Broth (LB) agar plates as described (Yao et al., 2013). The average number of cfu per

cm² was calculated. Log-transformed data were statistically analyzed. The data shown are representative of three independent experiments.

Plants were infected with *Botrytis cinerea* as previously described (Murmu et al., 2014). Spores of *B. cinerea* strain RW1A6P (Micalizzi et al., 2017) were harvested into sterile water 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 22°C were placed separately on three layers of moist sterile Whatman filter paper (Fisher Scientific, ON, Canada). The center of each leaf was inoculated with 6 µL of the spore suspension. The plates were sealed with surgical tape and incubated in the dark for the first 12 hours and then transferred to a growth chamber set at 22°C under long days. The diseased leaves were photographed to record lesion spread. Lesion sizes were measured from the photographs using ImageJ software (<http://www.nih.gov>). Data shown are means ± SE from 10 infected leaves using five plants per genotype. The data are representative of three independent experiments.

3.3.8 Seedling growth inhibition assay

Seedling growth inhibition assays were performed as previously described (Schwessinger et al., 2011). Seeds were germinated on MS agar plates with 1% sucrose under continuous light at room temperature. Four-day-old seedlings were transferred to 24-well culture plates (one seedling per well) containing 500 µl of liquid MS media ± 1 µM flg22 peptide per well (P6622, PhytoTechnology Laboratories, Lenexa, KS, USA). After ten days, seedlings were individually harvested, blotted dry, and weighed. Each replicate contained 10-12 seedlings. Percent growth inhibition was calculated by dividing the average fresh weight of mock versus flg22-treated seedlings. Two to four independent experiments were performed, and the results were averaged.

3.3.9 Histological detection of H₂O₂

H₂O₂ production was monitored by 3-3'-diaminobenzidine (DAB) staining as described (Thordal-Christensen et al., 1997). Briefly, ten-day-old seedlings grown on MS agar plates with 1% sucrose under continuous light were stained in 1 mg ml⁻¹ DAB solution pH 3.8 (D8001, Sigma-Aldrich, St. Louis, MO) overnight in the dark. Stained samples were fixed and cleared in a 3:1:1 solution of ethanol:lactic acid:glycerol (v/v/v). Cleared samples were stored in 60% glycerol prior to imaging in 70% ethanol under a stereomicroscope (SteRIO Discovery V20, Carl Zeiss, Oberkochen, Germany). Plants were scored for H₂O₂ production as indicated by brown staining.

3.3.10 Oxidative burst assay

ROS production from leaf tissue was measured by H₂O₂-dependent luminescence of luminol as described (Keppler, 1989; Brauer et al., 2016). Duplicate 4-mm leaf disks were collected from 8 plants per genotype and floated adaxial side-up in deionized water overnight in a 96-well plate. The next day, the water was replaced with 100 µl of detection buffer [17 µl ml⁻¹ luminol (Sigma-Aldrich, St. Louis, MO, USA), 20 µl ml⁻¹ horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) and 100 nm 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.

3.3.11 Callose staining

Callose staining was performed as previously described (Schenk and Schikora, 2015). Briefly,

samples were fixed and cleared overnight in a 1:3 solution of acetic acid/ethanol (v/v). The next day, samples were stained in 0.01% aniline blue in 150 mM K₂HPO₄ for 12 hours in darkness. The next day, samples were mounted in 50% glycerol and imaged under UV light using a compound microscope (Axio Imager M2, Carl Zeiss, Germany). 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.

3.3.12 Yeast two hybrid

Protein-protein interactions were assayed using a Matchmaker® GAL4-based yeast two-hybrid system (Clontech) and Gateway-compatible pGPKT7-DEST (bait) and pGADT7-DEST (prey) vectors modified from pGBKT7 and pGADT7-Res plasmids, respectively (Lu et al., 2010b). Full-length BOP1/BOP2 and TGA1/TGA4 proteins fused to the DNA-binding domain of yeast GAL4 were used as bait as previously described (Wang et al., 2019). The coding sequences of *WRKY* genes used as prey were cloned into Gateway entry vector pENTR™/D-TOPO™. All inserts were sequenced to confirm genuineness then transferred into pGADT7-DEST using Gateway LR Clonase II enzyme mix according to the manufacturer's instructions (Invitrogen). Bait and prey plasmid were co-transformed into yeast AH109 strain using a high-efficiency lithium acetate method (Gietz and Schiestl, 2007). Yeast transformed with bait and prey plasmids were identified by selection on synthetic defined (SD) media plates lacking leucine and tryptophan, named as “double drop-out” (DDO) media. Positive colonies were cultured in fresh DDO medium. Dilution series were spotted onto DDO medium or SD medium lacking leucine, tryptophan, and histidine, named as “triple drop-out” (TDO) medium plus 0-8 mM 3-AT (3-amino-1,2,4-triazole, Sigma-

Aldrich) for assessment of histidine reporter gene activity as previously described (Wang et al., 2019).

For the analysis of BOP1 functional domains, TGA1 and WRKY60 proteins fused to the DNA-binding domain of yeast GAL4 were used as bait. Truncated BOP1 preys: BOP1-BTB (amino acids 1-190), BOP1-DUF/ANK (amino acids 190-467) and BOP1-ANK (amino acids 240-467) obtained through PCR amplification were cloned into Gateway entry vector pENTRTM/D-TOPOTM. To create BOP1-ΔDUF (an internal deletion of amino acids 121-239 in the full-length protein) fragments containing the BTB/POZ domain (aa 1-120) and ANK domain (aa 240-467) were PCR-amplified then joined using an In-Fusion[®] HD cloning kit (Takara Bio) and ligated into pENTRTM/D-TOPOTM. The inserts were sequenced to confirm genuineness then transferred into pGADT7-DEST using an LR reaction. Primers used for cloning BOP1 variants are listed in Supplemental Table 3.

3.3.13 Accession Numbers

RNA-seq raw data generated from this study can be accessed at Gene Expression Omnibus under accession number GSE157422. Other sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *BOP1* (At3g57130), *BOP2* (At2g41370), *TGA1* (At5g65210), *TGA4* (At5g10030), *SARD1* (At1g73805), *CBP60g* (At5g26920), *WRKY6* (At1g62300), *WRKY18* (At4g31800), *WRKY31* (At4g22070), *WRKY38* (At5g22570), *WRKY40* (At1g80840), *WRKY60* (At2g25000), *WRKY62* (At5g01900), *WRKY70* (At3g56400), *GAPC* (At3g04120), *ACT1* (At2g37620), and *UBQ10* (At4g05320). Germplasm used included T-DNA insertion lines *bop1-3* (SALK_012994),

bop2-1 (SALK_075879), *tga1-1* (SALK_028212), *tga4-1* (SALK_127923) and *fls2-17* (Zipfel et al., 2004).

3.4 Results

3.4.1 Transcriptome of *BOP1*-overexpressing plants reveals elevated defense-related gene expression

Arabidopsis BOP1 and *BOP2* have nearly identical expression patterns and act redundantly in controlling development (Hepworth et al., 2005; Norberg et al., 2005; Chan et al., 2007; Jun et al., 2010; Khan et al., 2015). Dwarf stature is the most obvious consequence of overexpressing *BOP1* (Figure 3.1A) or *BOP2* (Norberg et al., 2005; Khan et al., 2015). Previous microarray analysis of *BOP1*-overexpressing (*BOP1-oe*) plants linked this phenotype to the increased expression of genes in JA, SA, and ET-related signalling pathways, consistent with an immune response (Khan et al., 2015). To further explore this link, Gene Ontology (GO) term analysis was used to categorize *BOP1-oe* differentially expressed genes (Supplemental Dataset 1). Approximately one-third of up-regulated genes (1106 out of 2928, 38%) were annotated as “stimuli-related” (Figure 3.1B, Supplemental Dataset 2). Of these “stimuli-related” genes, 700 were annotated as biotic stress-related (Figure 3.1C, Supplemental Dataset 2). Further classification showed that 291 of 700 genes sorted to various 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%) (Figure 3.1C, Supplemental Dataset 2). To test the robustness of this GO analysis, differentially expressed genes in *BOP1-oe* were compared to published microarray data sets using Gene Set Enrichment Analysis (GSEA) (Supplemental Figure 3.1). For PTI, a dataset that combined transcriptional changes in response to three different PAMPs

was used for comparison (Wan et al., 2008). For JA/ET-mediated plant defense, a dataset showing transcriptional changes in response to *Botrytis cinerea* infection was used for comparison (Windram et al., 2012). For SA-mediated plant defense, a dataset showing transcriptional changes in response to SA treatment was used for comparison (Blanco et al., 2009). Consistent with GO analysis, GSEA found a top enrichment score of 0.53 for the PTI dataset (Supplemental Figure 3.1). Thus, *BOP1* overexpression causes heightened expression of defense genes, with greatest support for genes involved in the PTI response.

3.4.2 *BOP1*-overexpressing plants show broad-spectrum resistance to pathogens

Plants with constitutive activation of defense genes often show resistance to diverse pathogens (van Wersch et al., 2016). To directly test if *BOP1-oe* mutants had increased resistance, leaves were infiltrated with hemi-biotrophic bacterium *P. syringae* pv. *tomato* (*Pst*) DC3000, which strongly elicits SA-dependent pathways leading to SAR (Kim et al., 2008b; Katagiri et al., 2002). Four days after infection, *BOP1-oe* leaves showed substantial resistance to *Pst* DC3000, as they harbored significantly lower bacterial titers compared to wild-type, whereas *bop1 bop2* mutants showed significantly higher bacterial titers indicating susceptibility (Figure 3.2A). As a further test, *BOP1-oe* plants were challenged with *Botrytis cinerea*, a necrotrophic fungal pathogen that elicits a strong JA/ET-dependent defense response (Windram et al., 2012). Detached leaves of wild-type, *BOP1-oe*, and *bop1 bop2* plants were infected with a suspension of spores in the center of each leaf. 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). By contrast, the lesion on *bop1 bop2* leaves progressed rapidly and reached a diameter (11.2 ± 0.7 mm) that was ~3-fold larger than wild type (Figure 3.2B). Thus, *BOP1* overexpression correlates with increased

resistance to diverse pathogens whereas *bop1 bop2* mutants were more susceptible particularly to *B. cinerea*.

3.4.3 PTI responses are impaired in *bop1 bop2* and *tga1 tga4* mutants

Clade I TGA1 and TGA4 function with BOP1/2 in development (Wang et al., 2019) but have a positive, more characterized role in PTI (Kesarwani et al., 2007; Wang and Fobert, 2013; Sun et al., 2018). This PTI response can be specifically assayed using the *hrcC*⁻ mutant of *Pst* DC3000 (*Pst hrcC*⁻), which is unable to deliver effectors into plant cells (Hauck et al., 2003). A prior study showed that the *tga1 tga4* mutant is compromised in defense against *Pst hrcC*⁻ and various hallmarks of PTI including oxidative burst, callose deposition, and PR1 protein secretion (Grennan, 2006; Wang and Fobert, 2013). To test if BOP1 and BOP2 also play a role in PTI responses, leaves of wild-type, *BOP1-oe*, *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* plants were infiltrated with *Pst hrcC*⁻. Four days after infiltration, leaves of *BOP1-oe* harboured significantly fewer bacteria compared to wild type (Figure 3.3A). By contrast, leaves of *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* mutants harboured similar titres of *Pst hrcC*⁻ that were significantly higher than wild type (Figure 3.3A) indicating an impaired PTI response.

Clade I TGAs contribute to an ROS burst upon pathogen perception (Boller and Felix, 2009; Wang and Fobert, 2013). To test if BOP1 and BOP2 also contribute to this burst, the production of active oxygen was measured in wild-type, *BOP1-oe*, *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* leaves elicited with flg22 using the *fls2* mutant as negative control (Figure 3.3B). An ROS burst was rapidly induced in wild-type plants, peaking at 4-5 minutes. No significant deviation was observed in *bop1 bop2* mutants, but ROS production was both reduced and delayed in *tga1 tga4* double and *bop1 bop2 tga1 tga4* quadruple mutants, reaching about two-thirds of the

wild-type maximum (Figure 3.3B; Supplemental Figure 3.2AB). *BOP1-oe* leaves generated a significantly higher amount of ROS compared to wild type, indicating that BOP1/2, though not essential for ROS production as shown for *bop1 bop2*, can promote this response when up-regulated. Steady-state hydrogen peroxide levels in 3,3'-diaminobenzidine-stained leaves of *BOP1-oe* compared to wild-type and *bop1 bop2* mutants showed little or no increase, suggesting that *BOP1-oe* plants exhibit a priming effect when stimulated with elicitor (Supplemental Figure 3.3A-C).

ROS signalling during PTI leads to multiple outputs, including the deposition of callose in the cell wall (Gómez-Gómez and Boller, 2000; Luna et al., 2011). To measure this response, leaves of wild-type, *BOP1-oe*, and mutants *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* with *fls2* as a negative control were infiltrated with *Pst hrcC* and flg22. Callose deposits were monitored after 24 hours by staining with aniline blue. A large number of callose deposits were observed after *Pst hrcC* challenge and flg22 treatment of wild-type leaves (Figure 3.3C). This response was significantly increased in *BOP1-oe* leaves, where callose deposits were 1.8-fold higher compared to wild type. Callose deposits in *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* leaves were significantly reduced to 67-80% of wild type levels (Figure 3.3C). No callose was deposited in *fls2* leaves treated with flg22 (Supplemental Figure 3.2C). Callose deposits in *BOP1-oe* non-treated plants were similar to wild type and *bop1 bop2* mutants except increased at leaf tips (Supplemental Figure 3.3D-I). These results show a specific role for BOP1/2 and clade I TGA factors in promoting callose under treatment conditions.

The initiation of a defense response by flg22 also reduces growth (Huot et al., 2014). The underlying mechanism may involve ROS-mediated stiffening of cell walls and negative BR

regulation (Belkhadir et al., 2014; Jiménez-Góngora et al., 2015; Neuser et al., 2019). We used a seedling growth inhibition assay (Gómez-Gómez et al., 1999) to measure this response in wild type, *BOP1-oe*, and mutants *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* using *fls2* as a negative control. Seedlings were grown on plates for 6 days and then transferred into liquid medium with or without flg22 elicitor. Seedlings were allowed to grow for another 10 days whereupon the average fresh weight of flg22-treated seedlings was compared to control seedlings and expressed as a ratio. Figure 3.3D shows that the growth of wild-type seedlings was reduced by 78% in the presence of elicitor. This inhibition was more pronounced in plants overexpressing *BOP1* where the addition of flg22 resulted a growth reduction of 94.8% compared to mock-treated seedlings of the same genotype. Conversely, *bop1 bop2* and *tga1 tga4* double mutants showed a diminished growth inhibition response compared to the 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 indicating additive effects of *bop1 bop2* and *tga1 tga4* in this response. Little or no growth inhibition was observed in *fls2* mutants, which are insensitive to flg22 (Supplemental Figure 3.2D). These collective data support a positive role for BOPs and clade I TGAs in repressing growth in response to elicitor.

3.4.4 BOP1/2 and clade I TGAs co-activate subset of defense genes in PTI

Substantially similar defects in *bop1 bop2* and *tga1 tga4* mutants in response to *Pst HrcC*⁻ and flg22 challenge led us to test if BOP1/2 and clade I TGAs co-regulate a subset of defense genes important for PTI. The expression of PTI-related defense genes was first measured in wild-type, *BOP1-oe*, and *bop1 bop2* stems and seedlings using RT-qPCR (Figure 3.4). Genes were chosen to represent diverse parts of the PTI response, ranging from pathogen perception to downstream

transcription factors and pathogenesis-related resistance genes (Supplemental Table 3.1). Consistent with the microarray, *BOP1-oe* stems showed higher transcript levels for the majority of selected PTI genes compared to wild type (Supplemental Table 3.1 and Figure 3.4A). Significantly higher transcript levels were recorded for flagellin receptor gene *FLS2* (Zipfel et al., 2004) and co-receptor gene *BOTRYTIS-INDUCED KINASE1 (BIK1)* (Lu et al., 2010a), which were up-regulated 3.0- and 4.5-fold, respectively. The respiratory burst oxidase gene *RESPIRATORY BURST OXIDASE HOMOLOG PROTEIN D (RBOHD)* responsible for ROS production (Morales et al., 2016) and callose synthase gene *GLUCAN SYNTHASE-LIKE 5* responsible for callose deposition (Jacobs et al., 2003) were also up-regulated in *BOP1-oe* stems by 1.3- and 1.8-fold, respectively. Significantly higher transcript levels were also recorded for clade I TGA-regulated genes *SARD1* (up-regulated 3.9-fold) and *CPB60g* (up-regulated 2.5-fold), which co-ordinate plant immunity (Sun et al., 2015, 2018). Trends observed in stems and seedlings were similar indicating that regulation of PTI-specific genes in *BOP1-oe* plants is not developmental stage- or tissue-specific (Figure 3.4AB). Transcripts in *bop1 bop2* tissues were generally similar or reduced compared to wild type (Figure 3.4AB).

We next tested if BOPs and clade I TGAs co-regulate a subset of these PTI genes under treatment with flg22. The induction of *SARD1*, *CBP60g*, *FLS2*, *RBOHD*, and *PR2* genes was measured in seedlings of wild-type and mutants *BOP1-oe*, *bop1 bop2*, *tg1 tga4*, and *bop1 bop2 tg1 tga4* using *fls2* as a negative control (Figure 3.5). As expected, all of these genes were significantly induced in the wild type after 4 hours of flg22 treatment and no induction was observed in *fls2* seedlings (Zipfel et al., 2004; Wang et al., 2009, 2011; Morales et al., 2016). Compatible with the microarray, all of these genes were expressed at significantly higher levels in *BOP1-oe* untreated seedlings at levels comparable to wild-type plants after flg22 treatment. The

most significant gene induction by flg22 treatment was observed in *BOP1-oe* mutants compared to base levels in wild-type plants. In particular, *SARD1* transcripts were dramatically induced by 4.8-fold after 4 hours of flg22 induction with similar induction of *CBP60g*. By contrast, transcript levels of *SARD1*, *CBP60g*, *FLS2*, *RBOHD*, and *PR2* were less responsive to flg22 elicitation in *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* mutants compared to wild type. These data suggest that BOP1/2 and clade I TGAs are required for the full activation PTI genes in response to flg22 treatment.

3.4.5 RNA sequencing reveals *BOP1-oe* transcriptome shifted towards defense and flg22-insensitive gene cluster in *bop1 bop2* and *tga1 tga4* mutants

To examine this finding on a more global scale, RNA sequencing was used to compare wild-type, *BOP1-oe*, *bop1 bop2*, and *tga1 tga4* seedlings treated with flg22. Transcripts from each genotype were sequenced in duplicate after 4 hours of exposure to 1 μ m flg22 or a mock solution. We first considered the overall pattern of gene expression for transcripts responsive to flg22 treatment. Using cluster analysis, we were able to completely separate the flg22-treated samples from control samples, except for *BOP1-oe* samples, which grouped towards flg22-treated wild-type samples (Figure 3.6A). The same trend was observed using Principal Component Analysis (PCA) (Figure 3.6B). The first two principal components demonstrated 69% and 18% of variance, respectively. Along the x-axis, PC1 discriminated four major groups: 1) mock-treated samples excluding *BOP1-oe*, 2) mock-treated *BOP1-oe*, 3) flg22-treated wild type and *BOP1-oe*, and 4) flg22-treated *bop1 bop2* and *tga1 tga4* samples. As seen with cluster analysis, untreated *BOP1-oe* samples were clearly shifted towards treatment groups and distinct from untreated control samples. Along the y-

axis, PC2 clearly separated mock and flg22-treated *BOP1-oe* samples from the other groups (Figure 3.6B).

An analysis of differentially expressed genes (DEGs) found that 1401 genes were significantly induced and 1241 genes were significantly repressed in untreated *BOP1-oe* samples compared to 2074 significantly induced and 873 significantly repressed genes in flg22-treated wild-type samples (Figure 3.6C; Supplemental Dataset 3). A GO analysis of induced genes in these datasets found extensive overlap as well as specific differences. 125 of 155 (81%) significantly GO-enriched terms in flg22-treated wild-type were also enriched in the *BOP1-oe* dataset demonstrating a significant overlap (Supplemental Dataset 4; Fisher's Exact Test, Bonferroni Correction, $p < 0.05$). Seven of the top 10 enriched terms in flg22-treated wild-type showed similar or higher fold-enrichment in *BOP1-oe* including “defense response by cell wall thickening”, “response to chitin”, and “regulation of JA-mediated signaling pathway”. GO terms related to the production of defensive compounds “indolalkylamine metabolic process” and “tryptophan metabolic process” were also highly enriched in both groups. Other prominent terms in flg22-treated wild-type like “pattern recognition receptor signaling pathway” and “defense response by callose deposition” were absent in the GO analysis of *BOP1-oe* where instead, terms related to specialized processes within PTI such as “camalexin biosynthesis process” and “phytoalexin biosynthesis process” showed high enrichment values (Figure 3.6C; Supplemental Dataset 4).

The overlap between *BOP1-oe* and flg22-treated wild-type datasets defined a group of 906 genes that were upregulated when *BOP1* was overexpressed under control conditions (Figure 3.6D; Supplemental Dataset 5). Within this group, 683 genes showed no further significant increase upon exposure to flg22 (Figure 3.6D). GO terms related to “indolalkylamine metabolic process” and

“tryptophan metabolic process” were significantly enriched by ~17-fold in this gene subset. Categories “defense response by cell wall thickening”, “regulation of JA-mediated signaling pathway” and “regulation of systemic acquired resistance” were also prominent (Supplemental Dataset 4).

To identify potential co-factors of BOP1 involved in upregulation of these 683 genes, the promoter regions (500 base pairs upstream of the transcription start sites) were scanned for the occurrence of all possible 6-mers (Figure 3.6E). Consistent with the involvement of TGA transcription factors, the *as-1*-like element “AAGTCA/TGACTT” was found one or more times in 285 of these 683 promoters (Krawczyk et al., 2002; Ding et al., 2018). Three WRKY transcription factor binding sites, “AGTCAA/TTGACT”, “AAAGTC/GACTTT”, and “GTCAAC/GTTGAC” (Birkenbihl et al., 2017; Chen et al., 2019b) were also overrepresented and ranked higher in both occurrence and significance, suggesting an unexplored role for WRKY factors in BOP1-dependent induction of defense genes.

Principal Component Analysis comparing the transcriptomes of *bop1 bop2* and *tga1 tga4* flg22-treated samples showed clustering that was distinct from wild type (Figure 3.6B). Of 2074 differentially upregulated genes in the wild type, about two-thirds were induced at significant levels in *bop1 bop2* and *tga1 tga4* samples (Figure 3.7A; Supplemental Datasets 3 and 5). The transcriptomes of *bop1 bop2* and *tga1 tga4* flg22-treated samples were quite similar, showing the induction of 1848 same genes versus 524 and 860 unique genes, respectively (Figure 3.7A). Notably, 378 significantly induced genes in wild-type showed little change in *bop1 bop2* and *tga1 tga4* samples indicating a low response to flg22 treatment (Figure 3.5 and 3.7AB; Supplemental Dataset 5). Among this list were clade I TGA-regulated *SARD1* and *CBP60g* genes, seven *WRKY*

transcription factor genes, *PHYTOALEXIN DEFICIENT 4* required for the production of phytoalexins (Glazebrook et al., 1997), two TGA-interacting CC-type glutaredoxin genes involved in redox-regulation (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 (O'Brien et al., 2012; Lee et al., 2019).

3.4.6 BOP1 directly binds to clade I TGA-regulated *SARDI* and *CBP60g* gene promoters

To probe the model that BOP1/2 and clade I TGA1/4 co-regulate defense genes, we used ChIP assays to test if BOP1 directly interacts with promoters of *SARDI* and *CBP60g*. Both of these TGA1/4-regulated genes, encoding calmodulin binding family-type transcription factors, are rapidly induced by pathogen infection (Wang et al., 2009; Zhang et al., 2010; Sun et al., 2018). Numerous motifs that match or closely match TGA consensus binding sites were found in the DNA sequences upstream of *SARDI* and *CBP60g* start codons (Figure 3.7C and 3.7D). Previous work showed that TGA1 directly activates *SARDI* whereas regulation of *CBP60g* might be indirect (Sun et al., 2018). We therefore used ChIP assays to investigate if BOP1 binds at TGACG motifs previously identified by Sun et al. (2018). The experiment was carried out using *BOP1p:BOP1-GR (GLUCOCORTICOID RECEPTOR) bop1 bop2* seedlings using an anti-GR antibody (Khan et al., 2015). Quantitative PCR on immunoprecipitated DNA template showed the significant enrichment of BOP1 at TGACG motifs in *SARDI* and *CBP60g* promoters but only in flg22-treated samples. Little or no binding above background was detected in non-stimulated samples (Figure 3.7E and 3.7F). This finding suggests that BOP1 binding to *SARDI* and *CBP60g* promoters is dependent on flg22 treatment.

3.4.7 WRKY transcription factors interact with BOP1/2 and clade I TGAs

Promoter analysis of *BOP1*-induced genes, including *SARD1* and *CBP60g*, found an enrichment of sequences that resemble WRKY binding sites (Figure 3.6E and Figure 3.7CD). Indeed, WRKY and TGA factors bind to similar core sequences (TGAC) and work synergistically and/or antagonistically in regulating gene expression (Maleck et al., 2000; Zhou et al., 2018; Ding et al., 2018; Hussain et al., 2018). In total, we identified 40 *WRKY* genes with differential expression in *BOP1-oe* datasets (Figure 3.8A and Supplemental Table 3.2). Six of these *WRKY* genes showed impairment of expression in *bop1 bop2* and *tg1 tg4 flg22*-treated samples (Figure 3.7B).

To test if BOP1/2 or clade I TGAs interact with WRKY proteins, we used yeast two-hybrid assays (Supplemental Table 3.2). BOP1/2 and TGA1/4 proteins fused to the DNA-binding domain of yeast transcriptional activator GAL4 were used as bait. Based on differential expression in our datasets and previously identified defense-related functions, a set of 22 WRKY proteins were fused to the transcriptional activation domain of GAL4 and tested as prey. Figure 3.8B shows that BOP1 interacted with WRKY6, 31, 60, and 62. BOP2 interacted with the same proteins, except for WRKY6 (Table 3.3). Clade I TGA interactions showed considerable overlap with BOP1 and BOP2 as TGA1 interacted with WRKY31, 38, 60, and 62 and weakly with WRKY6 and 18 (Figure 3.8B). TGA4 showed the greatest number of interactions, forming complexes with WRKY6, 38, 46, 60, 62, and 63 (Table 3.3). Interestingly, none of the baits interacted with WRKY70, which represses *SARD1* under resting conditions along with NPR4 and TGA2/5/6 but activates *SARD1* along with NPR1 and TGA2/5/6 in response to SA (Zhou et al., 2018; Ding et al., 2018).

Finally, we used deletion mutants of BOP1 to test which functional domains are required for TGA and WRKY binding (Figure 3.8C). The BOP1 and BOP2 proteins contain three conserved

motifs: a N-terminal BTB/POZ (BTB) domain, a central DUF3420 (DUF) domain of unknown function, and an adjacent ANKYRIN (ANK) repeat domain (Figure 3.8C; Khan et al., 2014). Full length and partial BOP1 proteins including: BOP1-BTB (amino acids 1-190), BOP1-DUF/ANK (amino acids 190-467), BOP1-ANK (aa 240-467) and BOP1- Δ DUF (amino acids 121-239 removed) as prey were tested for interaction with TGA1 and WRKY60 proteins fused to the DNA binding domain of yeast GAL4 as bait, respectively. Constructs BOP1-BTB, BOP1-ANK, and BOP1- Δ DUF failed to interact with either TGA1 or WRKY60. Only full length BOP1 protein and BOP1-DUF/ANK, which lacks the BTB-domain showed an interaction (Figure 3.8C). This result suggests that the combined DUF and ANK domains of BOP1 are important for TGA and WRKY binding.

3.5 Discussion

BTB-ankyrin proteins are conserved transcription co-factors in land plants (Khan et al., 2014). Subdivision of BTB-ankyrin proteins into BLADE-ON-PETIOLE (BOP)-like and NON-EXPRESSOR OF PR1 (NPR1)-like clades is described in mosses and higher plants (Saleh et al., 2011; Peng et al., 2017; Backer et al., 2019). NPR1-like members of this family perceive SA and regulate SAR whereas BOP-like members regulate plant development (Khan et al., 2014; Richardson and Hake, 2019; Backer et al., 2019). In brief, we describe a role for BOP1 and BOP2 involving clade I TGA and WRKY transcription factors in plant immunity. This role was uncovered through characterization of *BOP1-oe* plants, which exhibit activation of defense genes at the expense of growth. Categorization of these genes depicted a transcriptome for *BOP1-oe* mutants that substantially overlaps with wild-type plants treated with flg22. Consistent with this profile, *BOP1-oe* plants displayed increased immunity, production of ROS, and deposition of

callose whereas *bop1 bop2* and *tga1 tga4* mutants exhibited clear defects in PTI. Further analysis identified co-regulation of defense genes by BOP1/2 and clade I TGAs including *SARD1* and *CBP60g*. Whereas TGA1 binds to *SARD1* under resting conditions, ChIP assays showed that BOP1 recruitment to *SARD1* and *CBP60g* promoters requires an elicitor. This recruitment may depend on WRKY transcription factors, which we found to interact with BOP1/2 and TGA1/4 in yeast. These data provide evidence of a defense role for BOPs using functional partners similar to NPR1.

3.5.1 *BOP1-oe* plants prioritize defense over growth

BOP1 and BOP2 are co-transcription factors with enriched expression in organ boundaries (Chan et al., 2004; Jun et al., 2010; Norberg et al., 2005; Hepworth et al., 2005; Khan et al., 2012a). Boundaries are discrete zones that connect lateral organs to the plant body, characterized by cells that divide infrequently and have relatively stiff walls (Hamant et al., 2008; Aida and Tasaka, 2006b). BOP1 and BOP2 contribute to growth repression in boundaries as evidenced by *bop1 bop2* mutant phenotypes such as fused organs, leafy petioles, and increased complexity of flowers (Hepworth et al., 2005; Norberg et al., 2005; Chan et al., 2007). In contrast, plants with increased *BOP1* or *BOP2* are dwarf (Norberg et al., 2005; Khan et al., 2015). As shown in this study, the PTI response in *BOP1* overexpressing plants are primed under resting condition, which leads to prioritized defense response at the expense of growth.

A function of BOP1/2 in plant defense has long been speculated given a domain structure similar to NPR1 but direct evidence has been lacking (Hepworth et al., 2005; Khan et al., 2014; Wang et al., 2019). Initial characterization of *bop1 bop2* mutants showed a wild-type response to *P. syringae* infection (Hepworth et al., 2005; Canet et al., 2010). A possible role in JA-mediated

defense was implied by way of *bop1 bop2* defects in pathogen resistance induced by exogenous methyl jasmonate (Canet et al., 2012). Other studies demonstrated that NPR1 paralogs including BOP1 and BOP2 have various SA-binding capability *in vitro* (Castelló et al., 2018). All of these observations led us to further examine *BOP1-oe* plants for a defense phenotype.

A GO analysis of up-regulated genes in *BOP1-oe* plants determined by microarray revealed a large fraction of genes associated with biotic stress (Khan et al., 2015). Although genes from SA- and JA/ET defense pathways were represented, PTI-related genes were the most abundant, suggesting a role for BOP1 in this branch of plant immunity. Compared to wild-type plants, *BOP1-oe* plants exhibit enhanced resistance to *Pst HrcC* whereas *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* mutants were susceptible. The resistance of *BOP1-oe* was associated with a higher level of ROS and callose deposition upon elicitation with *Pst hrcC*- or flg22 but neither of these responses was markedly elevated in non-treated seedlings. RNA sequencing revealed that the *BOP1-oe* transcriptome was shifted toward that of wild-type plants treated with flg22. Thus, *BOP1-oe* plants partially fit the definition of autoimmunity (van Wersch et al., 2016).

The dwarf stature of *BOP1-oe* plants is corrected by inactivation of clade I TGA factors or downstream boundary genes known to function in the same pathway (Khan et al., 2012b, 2012a; Wang et al., 2019). It would be interesting to test if expression of defense genes is reduced under these conditions. *BOP1-oe* growth defects were previously linked to an increase in JA accumulation (Khan et al., 2015), which can repress growth in stems by antagonizing GA (Heinrich et al., 2013; Jang et al., 2020). However, knockout of a JA biosynthetic enzyme gene nor application of exogenous GA significantly recovered growth in *BOP1-oe* plants (Khan et al., 2015). Brassinosteroids (BR) are another group of hormones implicated in growth-defense trade-

offs. Activation of BR signaling leads to nuclear localization of BRASSINAZOLE-RESISTANT 1 (BZR1) and BRI-EMS SUPPRESSOR 1 (BES1), key transcription factors of the BR response (Clouse, 2011; Wang et al., 2013). BR signaling promotes growth but represses innate defenses (Lozano-Durán and Zipfel, 2015). Activation of BZR1 is sufficient to hinder PAMP-triggered ROS production, seedling growth inhibition, and gene expression, all leading to enhanced susceptibility of plants to strains of *P. syringae* (Fan et al., 2014; Lozano-Durán et al., 2013; Malinovsky et al., 2014). A chemical genomic screen revealed that BOP1 interacts with BZR1 and hinders its import from the cytoplasm to the nucleus, thus down-regulating BR responses (Shimada et al., 2015). Independently, BOP1 and BOP2 reinforce a BR minimum at meristem-organ boundaries by activating a transcription factor that promotes the expression of *BASI*, a cytochrome P450 gene that promotes BR breakdown (Bell et al., 2012; Chan et al., 2007). Testing whether BOP1 and BOP2 repress BR to restrict growth within innate immunity is an important future direction.

Growth-defense phenotypes generated by BOP1/2 loss or gain might also reflect changes in ROS homeostasis. ROS signaling is critical for immunity but causes cell wall stiffening through cross-linking and lignin formation (Schöpfer, 1996; O'Brien et al., 2012; Lee et al., 2013, 2019). Microarray analysis identified *RBOHD*, twenty peroxidases, and two laccases as significantly upregulated in *BOP1-oe* stems (Supplemental Data Set 1). No resting ROS increase was reproducible in seedlings, but *BOP1-oe* leaf disk assays showed a significantly stronger ROS burst compared to wild type upon treatment with a PAMP. Inversely, a number of enzyme genes that contribute to ROS processes (three respiratory burst oxidases, six laccases, and one peroxidase) showed lower induction in flg22-treated *bop1 bop2* seedlings, compatible with a reduced growth inhibition response. The growth inhibition response of *bop1 bop2 tga1 tga4* quadruple mutant was

weaker than in *bop1 bop2* and *tga1 tga4* double mutants suggesting additive impacts of BOP1/2 and clade I TGAs on this process.

3.5.2 BOP1/2 and clade I TGAs co-regulate a subset of PTI genes

Clade I TGAs act primarily in innate immunity but function in other defense pathways as well (Kesarwani et al., 2007; Shearer et al., 2012; Wang and Fobert, 2013; Després et al., 2003; Sun et al., 2018). Genetic analysis of *tga1 tga4 npr1-1* triple mutants and microarray analysis of SA-treated plants showed that these two TGA transcription factors regulate fewer genes than NPR1 and are not essential for SA-induced *PR* gene expression (Shearer et al., 2012; Wang and Fobert, 2013). Nevertheless, the induction of two master immunity transcription factor genes *SARD1* and *CBP60g* was impaired in *tga1 tga4* mutants, correlating with lower elevation of SA following *P. syringae* infection and susceptibility (Zhang et al., 2010; Wang et al., 2011; Sun et al., 2018). Our RNA sequencing depicted broad similarity between *tga1 tga4* and *bop1 bop2* mutants in response to flg22 (Figures 3.6AB and 3.7A). In particular, the double mutants showed lower induction *SARD1* and *CBP60g* (Figures 3.5 and 3.7B) and lower induction of free SA after flg22 treatment of seedlings compared to wild type (Supplemental Figure 3.4). ChIP-qPCR experiments found that BOP1 binds to a region enriched in TGA and WRKY binding elements close to the transcriptional start sites for *SARD1* and *CBP60g* but only in flg22-treated samples. Since *BOP1* and *BOP2* expression does not significantly increase under flg22 treatment (Supplemental Data Set 2; Denoux et al., 2008) a PTI-generated signal or modification might increase binding activity.

Translocation of NPR1 from the cytoplasm to the nucleus is an important regulatory step in SAR (Mou et al., 2003). Prior to infection, NPR1 is mainly found in the cytoplasm as an oligomer (Kinkema et al., 2000; Mou et al., 2003). The production of SA and subsequent oxidative

stress leads to the production of thioredoxins that act on redox-sensitive cysteine residues in NPR1 to reduce and partially dismantle the oligomer (Mou et al., 2003; Tada et al., 2008). Monomeric NPR1 is translocated to the nucleus (Mou et al., 2003; Kinkema et al., 2000; Maier et al., 2011). NPR1 has a bipartite nuclear localization sequence of which phosphorylation is also required for import and establishment of SAR (Lee et al., 2015). BOP1 and BOP2 have no obvious nuclear localization sequence but reside in the cytoplasm and nucleus, and may partition between these compartments (Hepworth et al., 2005; Jun et al., 2010; Wu et al., 2012a). The cytoplasmic fraction of BOP1-GFP appears as punctate spots *in vivo*. This morphology correlates with protein oligomers formed *in vitro* that decrease under reducing conditions as for NPR1 (Mou et al., 2003; Shimada et al., 2015). A study in cotton reported that GhBOP1 becomes concentrated in the nucleus after challenge with fungal pathogen *Verticillium dahliae* leading to the production of lignin (Zhang et al., 2019).

Post-translational modifications of NPR1 control its affinity for TGA and WRKY proteins (Chen et al., 2019a; Saleh et al., 2015). Upon SA induction, NPR1 is sumoylated. This sumoylation increases NPR1 binding with TGA3 but decreases affinity for WRKY70 (Saleh et al., 2015). ChIP experiments found that SA treatment shifts NPR1 association from W-box repressor sites to *as-1* enhancer elements in the *PR1* promoter. NPR1 phosphorylation at Ser55/Ser59 blocks sumoylation but promotes WRKY70 interaction showing a switch-like mechanism (Saleh et al., 2015). Another study *in vitro* found that SA promotes NPR1 interaction with WRKY18 and CYCLIN-DEPENDENT KINASE8. This kinase forms a complex with NPR1 and TGA5/TGA7 that facilitates recruitment of RNA polymerase II to the *PR1* promoter for activated expression (Chen et al., 2019a). BOP1 and BOP2 bind constitutively with clade I TGA factors but this binding is relatively weak (Hepworth et al., 2005; Wang et al., 2019). Modification of redox-sensitive

Cys260 and C266 residues in TGA1 strengthens an interaction with NPR1 (Lindermayr et al., 2010; Després et al., 2003) but not BOP1/BOP2 (Wang et al., 2019). A genetic complementation of the *tga1 tga4* mutant with a cysteine-abolished form of *TGA1* can rescue TGA1/TGA4-dependent gene expression indicating that redox-regulation of clade I TGAs might not be critical in plant immunity (Budimir et al., 2020).

3.5.3 BOP1 and BOP2 interact with WRKY transcription factors

This study demonstrates that BOP1/2 and clade I TGAs interact with WRKY transcription factors. Future experiments will test the significance of these interactions on immunity and growth. *WRKY* genes are widely induced by stress and function combinatorically (Eulgem and Somssich, 2007; Bakshi and Oelmüller, 2014). Of 74 *WRKY* genes in Arabidopsis, more than half, a total of 40 *WRKY* transcription factors, were up-regulated in *BOP1-oe* stems and/or seedlings. Selective interactions were observed. For example, BOP1 but not BOP2 interacts with *WRKY6*, which can activate or repress transcription under stress, senescence or defense programs (Robatzek and Somssich, 2002, 2001). Target genes under positive regulation by *WRKY6* include *FRK1* and *PR1* whereas *PHOSPHATE1* and *WRKY6* are repressed (Robatzek and Somssich, 2002, 2001; Chen et al., 2009). Overexpression of *WRKY6* results in dwarf plants with reduced apical dominance showing an effect on growth (Robatzek and Somssich, 2002; Chen et al., 2009). Both BOP1 and BOP2 interact with *WRKY31*, *WRKY60*, and *WRKY62* whose roles in defense are complex. Transgenic soybean expressing a *WRKY31* ortholog displayed enhanced resistance to oomycete infection and constitutive activation of *PR* genes (Fan et al., 2017). *WRKY60* physically and functionally interacts with *WRKY18* and *WRKY40* in modulating the repression of genes involved in ABA signaling (Chen et al., 2010; Antoni et al., 2011; Yan et al., 2013) and PAMP-

triggered basal defense (Xu et al., 2006; Shen et al., 2007). Triple mutant *wkry18 wrky40 wrky60* showed greater resistance to *Pst DC3000* and a powdery mildew pointing to a group role in repressing basal defense (Xu et al., 2006; Shen et al., 2007). Although this resistance did not result in constitutive expression of defense genes, genome-wide expression profiling of plants inoculated with *Pst DC3000* showed that at least 21 PAMP-responsive genes accumulated earlier and to a greater extent than in wild-type plants (Shen et al., 2007). WRKY62 along with WRKY38 are activated by NPR1 (Wang et al., 2006; Spoel et al., 2009) but have additive roles in repression of basal defense via interaction with Histone Deacetylase 19 (HDA19) (Kim et al., 2008a). Both of these WRKYs interact with TGA1 and TGA4 raising the possibility of higher-order complexes incorporating BOP1 or BOP2. We found that BOP1-TGA1 and BOP1-WRKY60 interactions require both the DUF3420 and ANK-repeat domains in BOP1. The DUF3420 domain (PFAM PF11900) is an upstream motif associated with the ANK repeat, possibly involved in stabilizing this surface for interactions with proteins. It remains unclear if BOP1/2 can interact simultaneously with both TGA and WRKY proteins.

3.5.4 BOP/TGA/WRKY combinatorial control

Biochemical studies show that BOP1/2 activate transcription when recruited to DNA (Jun et al., 2010; Khan et al., 2015). Independently, BOP1/2 are CUL3 adaptors for E3 ubiquitin ligase complexes involved in the degradation of LEAFY and PIF4 proteins (Zhang et al., 2017; Chahtane et al., 2018). A combination of these two mechanisms, transcriptional activation and removal of a repressor, may be involved in regulating defense genes. For instance, NPR3 and NPR4 partner with clade II TGA factors and co-repress SA-responsive genes including *SARD1* and *WRKY70* in the absence of infection (Ding et al., 2018). As well, NPR3 and NPR4 interact with E3 ligase

complexes and mediate NPR1 turnover in the nucleus (Fu et al., 2012). Turnover mediated by NPR4 is proposed to inhibit the spurious activation of defense genes since NPR4-NPR1 pairing *in vitro* is strongest under low SA conditions (Fu et al., 2012). At the same time, proteasome-mediated turnover of NPR1 is critical for the full induction of target genes and the establishment of SAR (Spoel et al., 2009). Biochemical studies on the *PR1* promoter show that TGA2 is a repressor in resting cells (Rochon et al., 2006). During a defense response, SA binds to NPR1 causing a conformational change that releases its C-terminal transactivation domain from inhibition (Wu et al., 2012b). TGA2 is converted from a repressor into an activator by incorporating NPR1 into the complex (Rochon et al., 2006; Boyle et al., 2009). *In vitro* studies show that WRKY50 binds to nearby W-box elements in the *PR1* promoter and interacts with TGA2 and TGA5. Co-expression of WRKY50 and TGA2/5 synergistically induces *PR1* expression in Arabidopsis protoplasts (Hussain et al., 2018). Interaction of WRKY53 and TGA3 on the *Cestrum yellow leaf curling virus* promoter (*CmYLCV*) has a similar synergistic effect on expression in SA-treated tobacco protoplasts (Sarkar et al., 2018). One report hints that WRKY proteins can initiate NPR1 binding to the *PR1* promoter (Saleh et al., 2015). ChIP assays showed a shift in NPR1 association from W-box repressor sites to *as-1* enhancer sites in the *PR1* promoter in SA-treated plants (Saleh et al., 2015). Similarly, on the *SARD1* promoter, WRKY70 and TGA2/5/6 maintain repression under normal conditions (Zhou et al., 2018; Ding et al., 2018). Incorporation of NPR1 into the complex under SA treatment induces gene expression (Ding et al., 2018). Future experiments will test how these interlocked scenarios apply to BOP1/2 and TGA/WRKY regulation of defense genes during immunity.

3.5.5 Dual functions in development and defense

Our studies point to a role for BOPs in reprioritizes plants for defense at the expense of growth. We also provide evidence that BOPs function with clade I TGA and WRKY transcription factors in this role. Unlike animal cells that are free to migrate, morphogenesis in plants relies on coordinated patterns of cell proliferation, expansion, and differentiation. Because plants lack mobile immune cells, each cell can arrest growth and switching to defense. Recent studies in a moss, *Physcomitrella patens*, showed that simultaneous disruption of all three *BOP* genes produced no obvious change in morphology, perhaps pointing to a more ancestral, physiological role in immunity (Hata et al., 2019). Our studies support this view, showing a role for BOPs in defense. Transcriptome data in *BOP1-oe* plants indicates this role that may not be specific to any one branch of plant immunity but holds significant overlap with innate immunity responses, which are a first layer of defense in reinforcing the outer barriers of a cell. We envision that these defense mechanisms especially the PTI responses that lead to repression of growth and fortification of cell walls overlap with processes that take place at boundaries (Belkhadir et al., 2014; Patharkar and Walker, 2019; Olsson et al., 2019). Future experiments will test these links and discover how BTB-ankyrin proteins interact to co-ordinate different layers of plant immunity. This knowledge may yield strategies for greater disease protection in crops without broader detrimental effects like dwarfism.

Table 3.1 Primers used in Chapter 3.

Gene name	AGI identifier	Primer Sequence (5'-3') Forward, Reverse
<i>FLAGELLIN-SENSITIVE 2 (FLS2)</i>	At5g46330	ACTCTCCTCCAGGGGCTAAGGAT; AGCTAACAGCTCTCCAGGGATGG
<i>BOTRYTIS-INDUCED KINASE 1 (BIK1)</i>	At2g39660	TAAGCCACTCCCATGGTTTC; CGTTGTAGTCCGCATCAAGTA
<i>RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD)</i>	At5g47910	CATGGTATCAAGCTCTACCTCAC; AACAGCCACCTTGATCATCTTA
<i>At1g51890</i>	At1g51890	CAGCTGTGAGTTCTCTAGATGG; GCGGAAACTTCATCTTGGTATG
<i>CALCIUM DEPENDENT PROTEIN KINASE 28 (CPK28)</i>	At5g66210	GGAAGTTCGAATGCACACGGGG; GCAGGGCTTGCTGCTCTCTGTG
<i>WRKY DNA-BINDING PROTEIN 33 (WRKY33)</i>	At2g38470	GTGGAAGCAAGACAGTGAGAG; TGCACTTGTAGTAGCTTCTTGG
<i>WRKY DNA-BINDING PROTEIN 40 (WRKY40)</i>	At1g80840	GAGAGTAGCTCAACGGATCAAG; CATCTTTCACAACGAGGGTAGT
<i>WRKY DNA-BINDING PROTEIN 53 (WRKY53)</i>	At4g23810	CACCAGAGTCAAACCAGCCATTAC; CTTTACCATCATCAAGCCCATCGG
<i>SAR DEFICIENT 1 (SARD1)</i>	At1g73805	TCAAGGCGTTGTGGTTTGTG; CGTCAACGACGGATAGTTTC
<i>CALMODULIN-BINDING PROTEIN 60g (CBP60g)</i>	At5g26920	AAGAAGAATTGTCCGAGAGGAG; GGCGAGTTTATGAAGCACAG
<i>FLG22-INDUCED RECEPTOR-LIKE KINASE 1 (FRK1)</i>	At2g19190	GCGCAAGGACTAGAGTATCTTC; ATCTGACCGCTTCCTTCAAC
<i>GLUCAN SYNTHASE-LIKE 5 (GSL5)</i>	At4g03550	GAGTACATTCAGGTGCGGAAG; CTGAAGAAATCAAGCCTGTGC
<i>PATHOGENESIS-RELATED PROTEIN 2 (PR2)</i>	At3g57260	ATCTCCCTTGCTCGTGAATCTC; TCGAGATTTGCGTCGAATAGG
<i>ARABIDOPSIS NDRI/HIN1-LIKE 10 (NHL10)</i>	At2g35980	GATCGAAGCTCATGCCTACTAC; CTAGACTGTCCGGCGTTAAA
<i>GLYCERALDEHYDE-3-PHOSPHATE (GAPC)</i>	At3g04120	TCAGACTCGAGAAAGCTGCTA; GATCAAGTCGACCACACGG
<i>ACTIN 1 (ACT1)</i>	At2g37620	CGATGAAGCTCAATCCAAACGA; CAGAGTCGAGCACAATACCG
<i>UBIQUITIN 10 (UBQ10)</i>	At4g05320	CACACTCCACTTGGTCTTGCCT; TGGTCTTCCGGTGAGAGTCTTCA

Table 3.2 Primers used in chromatin immuno-precipitation and yeast-two-hybrid assays.

	Description	Name	Primer sequence (5'-3')	Reference
ChIP-qPCR	<i>SARDI</i>	<i>proSARDI</i> 0.3-kb ChIP-F	GGAACCGTCCATTTGTCAAC	Sun et al., 2018
		<i>proSARDI</i> 0.3-kb ChIP-R	TTCGAAGAACGACAAAGGAAA	
	<i>CBP60g</i>	<i>proCBP60g</i> 0.15-kb ChIP-F	GTTTCACTGCTGCTTCGTCA	Sun et al., 2018
		<i>proCBP60g</i> 0.15-kb ChIP-F	GGCTGTTCCGAATCTTCATT	
Y2H	BOP1-BTB (aa 1-190)	BOP1-C-SYFP-F	CACCATGAGCAATACTTTTCAAGAA	This study
		BOP1-190-R	CTAAGTAGTCCATAATTGATGCAT	
	BOP1-DUF/ANK (aa 190-467)	BOP1-190F	CACCTGCTCTTACTTAATCGCCAAATC	This study
		BOP1-C-SYFP-R	CTAGAAATGGTGGTGGTGGTG	
	BOP1-ANK (aa 240-467)	BOP1-240-F	CACCACTTTAGACCTCGAAGACCAGA	This study
		BOP1-C-SYFP-R	CTAGAAATGGTGGTGGTGGTG	
	BOP1-ΔDUF (aa 1-120^240-267)	BOP1-C-SYFP-F	CACCATGAGCAATACTTTTCAAGAA	This study
		BOP1-120R	CTAACAATTTCGATCTTGGCTCGTG	
		BOP1-Del-DUF	CACGAGCCAAGATCGAATTGTACTTTA GACCTCGAAGACCAGA	
		BOP1-C-SYFP-R	CTAGAAATGGTGGTGGTGGTG	

Table 3.3 Summary of yeast-two-hybrid assays.

WRKY	AGI identifier	Microarray (<i>BOP1-oe</i> vs WT)	RNA-seq (<i>BOP1-oe</i> vs WT)	RNA-seq (<i>BOP1-oe</i> flg22-treated vs WT)	Test status (Y/N)	BOP1 interaction (Y/N)	BOP2 interaction (Y/N)	TGA1 interaction (Y/N)	TGA4 interaction (Y/N)
4	At1g13960	+							
6	At1g62300		+	+	Y	Y	N	N	Y
8	At5g46350			+	Y	N	N	N	N
9	At1g68150			+					
11	At4g31550	+	+	+					
15	At2g23320	+	+	+	Y	N	N	N	N
17	At2g24570	+		+					
18	At4g31800		+	+	Y	N	N	N	Y
21	At2g30590	+							
22	At4g01250		+	+	Y	N	N	N	N
25	At2g30250	+	+	+	Y	N	N	N	N
26	At5g07100		+	+	Y	N	N	N	N
28	At4g18170			+					
29	At4g23550		+	+					
30	At5g24110		+	+	Y	N	N	N	N
31	At4g22070			+	Y	Y	Y	Y	Y
33	At2g38470	+	+	+	Y	N	N	N	N
35	At2g34830	+							
38	At5g22570	+	+	+	Y	N	N	Y	Y
39	At3g04670			+					
40	At1g80840	+	+	+	Y	N	N	N	N
41	At4g11070			+					
45	At3g01970		+	+	Y	N	N	N	N
46	At2g46400	+	+	+	Y	N	N	N	Y
48	At5g49520		+	+	Y	N	N	N	N
50	At5g26170	+							
51	At5g64810	+	+	+	Y	N	N	N	N
55	At2g40740		+	+					
57	At1g69310	+							
58	At3g01080		+	+					
59	At2g21900			+					
60	At2g25000		+		Y	Y	Y	Y	Y

61	At1g18860		+	+					
62	At5g01900	+		+	Y	Y	Y	Y	Y
63	At1g66600		+	+	Y	N	N	N	Y
67	At1g66550			+					
69	At3g58710	+							
70	At3g56400	+	+	+	Y	N	N	N	N
71	At1g29860		+	+	Y	N	N	N	N
75	At5g13080	+	+	+	Y	N	N	N	N

+, upregulated; Y, yes; N, no

Figure 3.1 Phenotype and transcriptome analysis of *BOP1*-overexpressing transgenic plants.

(A) Photographs showing wild-type and dwarf *BOP1-oe* plants used for microarray experiment. (B) GO enrichment analysis found that 1106 of 2928 differentially-induced genes ($\geq 0.5\log_2$ -fold, $p < 0.05$) in *BOP1-oe* stem were classified as “response to stimulus” (Supplemental Data Sets 1 and 2). (C) Stimuli-related genes were further categorized by GO ontology as response to biotic stimulus, abiotic stimulus, or general stress stimulus (Supplemental Data Set 2). (D) Biotic stimulus genes in *BOP1-oe* plants sorted into defense categories as follows: 162 PTI-related genes; 145 JA/ET-related genes, and 98 SA-related genes (Supplemental Data Set 2). Genes were sorted using PANTHER against the *Arabidopsis thaliana* TAIR11 genome annotation using default parameters (Fisher’s Exact Test, Bonferroni Correction, $p \leq 0.5$ as significant)

Figure 3.2 Growth of *Pst* DC3000 and *B. cinerea* in wild-type, *BOP1-oe*, and *bop1 bop2* plants.

(A) Leaves of four-week-old plants grown under short days were syringe-infiltrated with *Pst* DC3000 at 10^5 colony forming units (cfu) ml⁻¹. Log-transformed data are means $\pm$ SD of three replicates, each containing four leaf disks from separate plants. Three trials were performed, one is shown. (B) Detached leaves of four-week-old plants grown under long days were infected with 6 μ l of *B. cinerea* spores (5×10^5 spores ml⁻¹) and photographed as indicated for measurement of lesion size. Data are means $\pm$ SE from 10 infected leaves using five plants per genotype. Means with different lowercase letters are significantly different ($p \leq 0.01$, one-way ANOVA with Tukey's post-hoc test). The experiment was repeated twice, each time yielding similar results.

Figure 3.3 PTI response is enhanced in *BOP1-oe* and impaired in *bop1 bop2*, *tga1 tga4*, and *bop1 bop2 tga1 tga4* mutant plants compared to the wild-type.

(A) Growth of *Pst hrcC* in WT and mutant 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 $\pm$ SD of three replicates, each containing three leaf disks from separate plants. Means with different lowercase letters are significantly different ($p \leq 0.01$, one-way ANOVA with Tukey's post-hoc test). Data are representative of three trials. (B) flg22-induced oxidative burst in WT and mutant plants. Leaf discs (in duplicate) from four-week-old plants of the indicated genotypes were treated with 100 nm flg22 in the presence of luminol which reacts with H_2O_2 to produce light that was quantified in relative light units (RLU). The control treatment used water. Data are the mean $\pm$ SE of eight replicates per genotype. The *fls2* mutant was used as a negative control (Supplemental Figure 2). The experiment was repeated twice with similar results. (C) *Pst hrcC* and flg22 induced callose deposition in WT and mutants. Four-week-old leaves were infiltrated with water, 10^5 cfu ml^{-1} of *Pst hrcC* or 0.1 μM flg22 in water. Numbers at the top right corner of each panel indicate average callose counts $\pm$ SD per view. Three leaves from three independent plants were sampled per genotype. The *fls2* mutant was used as a negative control (Supplemental Figure 2). Data are representative of three trials. Asterisks, significant difference compared to WT control ($p \leq 0.001$, Student's t-test). Scale bar = 0.1 mm. (D) Quantification of seedling growth inhibition for WT and mutants exposed to 1 μM flg22. Data show percent growth of flg22-treated seedlings compared to control group. The *fls2* mutant used as negative control showed little or no growth inhibition response (Supplemental Figure 2). Data shown are means $\pm$ SD for 3 biological replicates. 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 3.4 Upregulation of PTI-associated defense genes in *BOP1-oe* transgenic plants.

(A-B) Transcript levels of genes characteristic of a PTI defense response were monitored in wild-type (WT), *BOP1-oe*, and *bop1 bop2* plants using RT-qPCR. For a description of genes and fold-change in microarray, see Table 3.2. Values were normalized to wild-type transcript levels in (A) stem of flowering plants and (B) 10-day-old seedlings. Plants were grown under continuous light. Internodes were harvested from the primary stem of soil-grown plants using 4-5 plants per replicate. Seedlings were grown on agar plates using 10-12 seedlings per replicate. Data shown are the mean $\pm$ SD of three biological replicates. Asterisks, significantly up, and triangles, significantly down, compared to wild type ($p \leq 0.5$, ANOVA with Tukey's post-hoc test).

Figure 3.5 BOPs and clade I TGAs co-regulate a subset of PTI genes.

(A-E) Relative transcript levels of selected PTI defense genes at 0 hours and 4 hours following treatment with 1 μ m flg22 elicitor. Values are shown relative to WT 0 h. Data are means $\pm$ SD of three biological replicates normalized to the reference genes *UBQ10* and *ACTIN1*. Each replicate contained 8-12 10-day-old seedlings grown on full-strength MS agar plate under continuous light condition. Asterisks indicate significantly different values between 0 and 4 hours (* $p \leq 0.05$, ** $p \leq 0.001$; one-way ANOVA with Tukey's post-hoc test).

Figure 3.6 Transcriptome of *BOP1-oe* mutant partially mimics the PTI response of flg22-treated wild-type seedlings.

(A) Gene-based clustering analysis of RNA-seq shows the sample-to-sample distance. Two biological replicates were used for each genotype and treatment. Arabidopsis seedlings were germinated on MS agar plates supplemented with 1% sucrose under continuous light. Four days after germination, seedlings were transferred to 24-well tissue culture plates containing 500 μ l of liquid MS media containing 1% sucrose per well. After another ten days, the culture solution was replaced with fresh liquid media with or without 1 μ M flg22 peptide for 4 hours. Each biological replicate was prepared from approximately 10 seedlings. Total RNAs were then sequenced using Illumina HiSeq X Ten. Further transcriptomic analysis was performed using DESeq2 (Ver. 2.11.40.6) implemented in Galaxy. Blue shading indicates similarity: the darker the blue, the more similar the samples. (B) Principal Component plot of transcriptome data derived from all samples. *x* and *y* axes indicate PC1 and PC2 respectively, and the proportion of variance for each principal component is shown in parentheses. Analysis performed using DESeq2 (Ver. 2.11.40.6) implemented in Galaxy. (C) Heat map showing fold-enrichment values for top 10 GO terms among differentially expressed genes (DEGs) up-regulated in WT treated with flg22 compared to untreated *BOP1-oe* samples. See also Supplemental Data Sets 3 and 4. (D) Venn diagrams depicting overlap between DEGs upregulated in WT treated with flg22 versus *BOP1-oe* under control and flg22 treatment conditions. See also Supplemental Data Sets 3 and 5. (E) Promoter analysis of DEGs in *BOP1-oe* samples showing flg22-independent induction. Statistical motif analysis was carried out using the 500 bp upstream of the start codon of each gene using the motif finder tool at TAIR. Enriched oligomers were matched to transcription factor binding sites as reported in literature. WRKY binding W-box, WT box, and W-box like sites (Birkenbihl et al., 2017; Chen et al., 2019b), TGA binding *as-1* like site (Ding et al., 2018) and starch box (Michael et al., 2008).

E

Motif name	Oligomer	Sequences containing oligomer (out of 683)	P-value	Known binding factors
W box	AGTCA/TTGACT	331	5.73e-21	WRKY
WT box	AAAGTC/GACTTT	311	8.27e-17	WRKY
W box-like	GTCAAC/GTTGAC	210	1.04e-13	WRKY
as-1 like	AAGTCA/TGACTT	285	4.12e-12	TGA
starch box	AAGCCC/GGGCTT	48	8.44e-12	unknown

Figure 3.7 Candidate target genes of BOP1/2 and clade I TGAs identified by transcriptome analysis and ChIP assays showing that BOP1 binds to the promoter region of *SARD1* and *CBP60g*.

(A) Venn diagrams showing overlap between flg22-induced genes in WT, *bop1 bop2*, and *tgal tga4* samples based on RNA sequencing (Supplemental Data Set 2). Further analysis focused on 378 genes showing induction in WT but not *bop1 bop2* or *tgal tga4* mutants. Also see Supplemental Data Set 2. (B) Candidate genes of interest with impaired induction in flg22-treated *bop1 bop2* and *tgal tga4* samples (Figure 3.5; Supplemental Data Set 2). (C-F) Binding of BOP1-GR to the promoter region of *SARD1* and *CBP60g* as determined by ChIP and RT-qPCR. Promoter maps (1.5 kb upstream of the start codons) above histograms show the location of predicted TGA bZIP (Schindler et al., 1992; Izawa et al., 1993; Fode et al., 2008; Sun et al., 2018) and WRKY (Ciolkowski et al., 2008; Machens et al., 2014; Maleck et al., 2000) binding sites in the promoter of *SARD1* and *CBP60g* in relation to fragments amplified by RT-qPCR (horizontal bars). Two-week-old seedlings grown in liquid medium were used for ChIP assay. A commercially available anti-GR antibody was used to pull-down genomic DNAs derived from mock and DEX-treated *BOP1p:BOP1-GR bop1 bop2* seedlings $\pm 1 \mu\text{M}$ flg22 grown under continuous light. Enrichment values were calculated as a percentage of input DNA of normalized to no-antibody control samples. Data represent the mean $\pm$ SE of three biological replicates, each performed using three technical replicates. Means with different lower-case letters are significantly different ($p \leq 0.05$, one-way ANOVA with Tukey's post hoc test).

B

WRKY factors		Glutaredoxins	
AT5G22570	WRKY38	AT5G14070	ROXY2
AT5G64810	WRKY51	AT4G15660	ROXY15
AT2G40750	WRKY54		
AT3G01080	WRKY58	Oxidative burst	
AT2G21900	WRKY59	At5G47910	RBOHD
AT5G01900	WRKY62	AT1G09090	RBOHB
AT3G56400	WRKY70	AT4G25090	RBOHG
		AT2G38080	LAC4
Innate Immunity		AT3G09220	LAC7
AT5G46330	FLS2	AT5G01040	LAC8
AT3G52430	PAD4	AT5G01190	LAC10
AT1G73805	SARD1	AT5G03260	LAC11
AT5G26920	CBGP60g	AT5G60020	LAC17
AT3G57260	PR2	AT5G51890	PRX66

Figure 3.8 WRKY transcription factors interact with BOP1 and TGA1.

(A) Venn diagrams showing intersection of up-regulated *WRKY* genes in *BOP1-oe* microarray (internodes of flowering plants) and RNA-seq (2-week-old seedlings) datasets under resting and flg22-treated conditions. Refer to Table 3.3. (B) Pairwise yeast two-hybrid assays showing BOP1 and TGA1 interaction with selected WRKY transcription factors. BOP1 and TGA1 baits were fused to the DNA-binding domain of yeast GAL4. WRKY preys were fused to the transcriptional activation domain of yeast GAL4. Plasmid constructs were co-transformed into yeast AH109 strain before serial dilutions (10^{-1} , 10^{-2}) were plated onto SD/-Trp/-Leu (DDO) medium. Growth on SD/-Trp/-Leu/-His + 8mM 3-AT (TDO) above background confirms a protein-protein interaction. The interaction of BOP1 with TGA1 was used as a positive control and an empty prey vector was used as a negative control. (C) Pair-wise yeast two-hybrid assays showing DUF and ANK domains are both required for interaction of BOP1 with TGA1 and WRKY60. Schematic maps showing full-length and truncated versions of BOP1 protein tested. A full-length BOP1 protein (1-467 aa) was used as a control. BOP1 can be divided into three major parts: 1-190 aa containing the BTB/POZ domain, 190-240 aa containing the DUF domain, and 240-467 aa containing the ANK domain. TGA1 and WRKY60 baits were fused to the DNA-binding domain of yeast GAL4. Truncated versions of BOP1 used as prey were fused to the transcriptional activation domain of yeast GAL4. Pair-wise interactions were assayed as above.

A **B** **C**

Supplemental Figure S3.1 Gene set enrichment analysis (GSEA) for *BOP1-oe* microarray compared to datasets representative of PTI, JA/ET, and SA-mediated defense responses.

For PTI, a dataset obtained by treatment of seedlings with three different PAMPs (flg22, elf26, and chitooctaose) was used for comparison (Wan et al. 2008). Enrichment score, 0.53; For JA/ET, a dataset showing transcriptional changes in response to *Botrytis cinerea* infection was used for comparison (Windram et al., 2012). Enrichment score, 0.22; For SA, a dataset of genes rapidly induced by SA treatment was used (Blanco et al. 2009) was used for comparison. Enrichment score, 0.45. *BOP1-oe* total DEGs were pre-ranked (organized based on log₂-fold change and p-value significance) and compared to the above datasets. GSEA 4.0.0 (a joint project of UC San Diego and Broad Institute) was used for the analysis.

A

BOP1-oe versus
PAMP-induced dataset
(Wan et al., 2008)

Enrichment score = 0.53

B

BOP1-oe versus
Botrytis-induced dataset
(Windram et al., 2012)

Enrichment score = 0.22

C

BOP1-oe versus
SA-induced dataset
(Blanco et al., 2009)

Enrichment score = 0.45

Supplemental Figure S3.2 PTI response assays for *fls2* mutant plants.

(A-B) flg22-induced oxidative burst in *fls2* plants was abolished. Leaf discs (in duplicate) from four-week-old plants of the indicated genotypes were treated with (A) 100 nM flg22 or with (B) H₂O in the presence of luminol which reacts with H₂O₂ to produce light that was quantified in relative light units (RLU). Data are the mean $\pm$ SE of eight replicates per genotype. (C) Callose deposition in *fls2* mutant leaves. Four-week-old leaves were infiltrated with 0.1 μ M flg22 or water control. After 12 hours, leaves were harvested, fixed and stained with aniline blue. Little or no callose deposition was observed. Scale bar = 0.1 mm. (D) The *fls2* mutant is insensitive to flg22-induced growth inhibition. Quantification of seedling growth inhibition for WT and *fls2* mutant exposed to 1 μ M flg22. Data show percent growth of flg22-treated seedlings compared to control group. Data show the mean $\pm$ SD for 3 biological replicates. Each replicate contained $n \geq 10$ seedlings.

Supplemental Figure S3.3 ROS and callose deposition in under resting conditions in WT, *BOP1-oe*, and *bop1 bop2* seedlings.

(A-C) Ten-day-old seedlings were stained with 3-3'-diaminobenzidine (DAB) for the detection of H_2O_2 . Resting ROS levels in *BOP1-oe* seedlings was similar to the wild-type in the majority of samples. Scale bar = 2 mm. (D-F) Leaves of 10-day-old seedlings stained with aniline blue for the detection of callose. Little or no callose was observed in the central area of leaves in the majority of samples. Scale bar = 100 μ m. (G-I) Leaf tip of 10-day-old seedlings stained with aniline blue for the detection of callose. Callose deposits at the tips of leaves were frequent in *BOP1-oe* but relatively rare in WT and *bop1 bop2* samples. Scale bar = 100 μ m. Numbers in the top right of panels refer to the number of samples displaying the phenotype shown.

Supplemental Figure S3.4 Flg22-elicited free salicylic acid (SA) accumulation in WT, *bop1 bop2*, and *tga1 tga4* mutants.

Four-week-old plants grown at 21-22°C under neutral light (12 h light/12 h dark) with a light intensity of $\sim 115 \mu\text{mol m}^{-2} \text{sec}^{-1}$ were infiltrated with *P. syringae hrcC* at a dose equivalent to an optical density at 600 nm of 0.05. Samples were harvested at 0 h (control) and 12 h after infiltration. A box plot representing mean $\pm$ SD of at least three independent biological samples is shown.

CHAPTER 4

BLADE-ON-PETIOLE 1 regulation of Arabidopsis defense is substantially independent of NONEXPRESSOR OF PATHOGENESIS- RELATED GENES 1

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4.1 Abstract

The BLADE-ON-PETIOLE1 (BOP1) and BOP2 belong to the NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) family of transcription co-factors. Members of this family are structurally similar yet surprisingly diverse in function. NPR1 and its three close homologs form a subclade with roles in the regulation of salicylic acid-mediated plant defense responses. BOP1 and BOP2 form another subclade. Initial characterization of BOP1 and BOP2 showed major roles in plant development but this role is not exclusive. Chapter 3 shows that BOP1 and BOP2 are positive regulators of pattern-triggered immunity (PTI). Interestingly, emerging evidence shows that members from different clades can form heterodimers indicating a potential for crosstalk between NPR1 and BOP1/2 clades. In this chapter, a null allele of *NPR1* (*npr1-3*) was introduced into a transgenic plant overexpressing *BOP1* (*BOP1-oe*) that exhibits enhanced immunity. Characterization of *BOP1-oe npr1-3* plants showed a slight recovery of dwarf stature. Rosette leaves in the double mutants may have a higher basal level of reactive oxygen species, which is consistent with a smaller rosette diameter. Further characterization of these plants found little or no effect of *npr1-3* on BOP1-regulated innate immunity. Moreover, the induction of several key genes in the PTI response, including *SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1* and *CALMODULIN BINDING PROTIEN 60g* was not altered. These data reveal that BOP1 function is substantially independent of NPR1.

4.2 Introduction

Plants have a complex hierarchy of defenses to combat pathogens (reviewed in Zhou and Zhang, 2020). When a pathogen is recognized by the receptors located on the cell membrane, a localized pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) response is first initiated (Macho and Zipfel, 2014; Bigeard et al., 2015; Tsuda et al., 2009). Some pathogens have evolved mechanism to bypass this level of plant immunity by injecting effectors into the plant cell to repress receptors that trigger PTI response. Plant cells can then recognize these effectors and mount a second layer of immune response called effector-triggered immunity (ETI) (reviewed in Tsuda et al., 2009). The ETI will eventually lead to a long-lasting systemic defense response in the distal parts of a plant called systemic acquired resistance (SAR) (Pieterse et al., 1998; Kinkema et al., 2000; Gao et al., 2015). The major outcomes of these different layers of defense responses are varied. For instance, the PTI response results in a rapid burst of reactive oxygen species leading to reinforcement of cell walls by callose deposition (Bigeard et al., 2015; Macho and Zipfel, 2014; Van Der Ent et al., 2009; Lee et al., 2013; O'Brien et al., 2012). The outcome of ETI is more severe with lesions of programmed cell death activated at the site of attack (Govrin and Levine, 2002; Lee et al., 2015; Keppler, 1989; Thordal-Christensen et al., 1997). On the other hand, SAR serves as a priming mechanism to heighten the defense response in distal parts of a plant against potential future pathogen invasions (reviewed in Fu and Dong, 2013). Recent advances indicate overlaps between different immune responses, which are tightly controlled under different phytohormones including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). In all cases, NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) is a master regulator which encodes a plant specific BTB-ankyrin protein that functions as a transcriptional co-factor (reviewed in Withers and Dong, 2016).

BTB-ankyrin proteins are characterized by an N-terminal BTB/POZ (Broad-Complex, Tramtrack, and Bric-a-brac/POX virus and zinc finger) domain and an internal ankyrin repeat domain (Godt et al., 1993; Mosavi et al., 2004; Chan et al., 2004; Norberg et al., 2005; Zhou et al., 2000; Zhang et al., 1999; Després et al., 2003). Each of these domains participates in protein-protein interactions (Godt et al., 1993; Mosavi et al., 2004). In *Arabidopsis thaliana*, NPR1 and its close homologs (NPR2, NPR3, and NPR4) form a primary subclade that functions in plant defense (Yan and Dong, 2014; Castelló et al., 2018; Fu et al., 2012). BOP1 and BOP2 form a second subclade that functions in plant development (Hepworth et al., 2005; Chan et al., 2007; Norberg et al., 2005) but also pattern-triggered immunity (Chapter 3). Heterodimers can be formed among these proteins through the BTB domain (Boyle et al., 2009; Castelló et al., 2018; Fu et al., 2012). The significance of these pairings is only partly understood.

NPR1 was identified by screening for *Arabidopsis* mutants lacking a proper SAR response (Cao et al., 1997; Glazebrook et al., 1996). All members of the NPR1 subclade bind SA molecules. Different affinities for SA binding regulate the formation of heterodimers that confer tight regulation of the immune response (Fu et al., 2012; Ding et al., 2018; Castelló et al., 2018; Glazebrook et al., 1996). During a defense response, NPR1 directly binds with SA molecule through two cysteine residues located at the C-terminal. This binding results in a conformational change that releases the transactivation domain so that NPR1 protein can activate defense genes (Wu et al., 2012b). On the other hand, NPR3/NPR4 are co-repressors when bound to DNA (Ding et al., 2018) and negatively regulate NPR1 stability by serving as adapters to Cullin3-based ubiquitin ligases (Fu et al., 2012). NPR4, which has the highest binding affinity for SA, binds with NPR1 under resting conditions when SA is low to inhibit SAR (Fu et al., 2012; Fu and Dong, 2013; Ding et al., 2018; Liu et al., 2020). During an ETI response, a high level of SA at the site of attack

results in an interaction between NPR3 and NPR1, thereby lowering NPR1 abundance and maintaining localized programmed cell death (Fu et al., 2012; Saleh et al., 2015). Recent studies suggest a broader role for NPR1 in pattern-triggered immunity. The loss-of-function *npr1-1* mutant was susceptible to *P. syringae* DC3000 *hrcC*⁻ mutant which triggers a PTI response in Arabidopsis (Ding et al., 2018). It was also shown that *npr1-1* cannot mount a successful defense against *P. syringae* DC3000 after priming with flg22 (Liu et al., 2020).

Although BOP1/2 are diverged from NPR1 and NPR1-like proteins, heterodimers can be formed between members of different subclades. Given the prominent role of NPR1 in plant immunity, we investigated the involvement of NPR1 in BOP1/2-mediated defense. Our data reveal that the defense function of BOP1 is substantially independent of NPR1.

4.3 Materials and methods

4.3.1 Plant material and growth condition

For phenotypic study, plants were grown in a cabinet at 21°C under short daylight (8h day/16 h night). Seedlings were first germinated on minimal media agar plates (Haughn and Somerville, 1986) for five days then transferred onto soil. Wild-type was the Columbia (Col-0) ecotype of Arabidopsis. The BOP1-overexpressor (*BOP1-oe*) and NPR1 null mutant *npr1-3* have been described previously (Norberg et al., 2005; Cao et al., 1997). The homozygous allele of *npr1-3* was confirmed with derived cleaved amplification polymorphism sequence (dCAPS). Primers used for dCAPS were published by Ding et al. (2015). The *BOP1-oe npr1-3* double mutant was constructed by crossing and confirmed by genotyping.

4.3.2 Bimolecular inflorescence complementation (BiFC) assay

A Gateway-compatible BiFC system was employed to test protein-protein interactions as described in section 2.3.4. Briefly, donor vectors for yeast two-hybrid were used for LR recombination into BiFC vectors to obtain *nYFP-BOP2*, *cYFP-PAN*, and *cYFP-NPRI* fusions, respectively. BiFC constructs were introduced into *A. tumefaciens* GV3101 by electroporation. Leaves of six-week-old *N. benthamiana* plants were infiltrated as described previously (Li et al., 2009). Samples were imaged using a Carl Zeiss LSM800 Airyscan confocal laser scanning microscope four days after infiltration. Images were processed using Zen 2.3 software (Carl Zeiss).

4.3.3 Histological staining of H₂O₂

Six-week-old seedlings grown under short daylight (8h day/16h night) were stained in 1 mg ml⁻¹ DAB solution pH 3.8 (D8001, Sigma-Aldrich, St. Louis, MO) overnight in the dark (Chapter 3.3.9). Stained samples were fixed and cleared in a 3:1:1 solution of ethanol:lactic acid:glycerol (v/v/v). Cleared samples were stored in 60% glycerol prior to imaging in 70% ethanol under a stereomicroscope (SterIO Discovery V20, Carl Zeiss, Oberkochen, Germany). Ten leaves per genotype, randomly collected from different plants, were stained and scored for H₂O₂ production as indicated by brown staining.

4.3.4 RT-qPCR

Total RNA was isolated from 10-day-old seedlings (10-15 seedlings per replicate) using a Plant Total RNA Mini Kit according to the manufacturer's instructions (Geneaid Biotech, Froggabio, Toronto, ON). The extracted total RNAs were treated with DNase I to remove residual genomic DNA as instructed by the manufacturer (Froggabio, Toronto, ON). The cDNA was synthesized

using 1 µg of total RNA with Superscript III 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. RT-qPCR reactions were performed in triplicate using Invitrogen Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Markham, ON) and run on an Applied Biosystems StepOnePlus thermocycler (Thermo Fisher Scientific, Markham, ON). Gene-specific primers were as listed in Table 4.1. Differences in gene expression, expressed as a fold-change relative to the wild type, were calculated as described (Pfaffl, 2001). Values were normalized to the reference genes *UBIQUITIN10* and *TUBULIN4*.

4.3.5 Pathology test

Plants were assayed for resistance against *P. syringae* pathovar (pv) *tomato* DC3000 *hrcC* mutant (*Pst HrcC*) which lacks a functional effector delivery system (Shearer et al., 2012). The protocol of *P. syringae* infiltration was modified from Liu et al. (2015). Briefly, bacteria were grown at 30°C on King's *B* medium containing Rifampicin (100 mg l⁻¹) and kanamycin (25 mg l⁻¹). Overnight cultures were resuspended in infiltration medium (10 mM MgCl₂). Leaves of four-week-old plants grown under neutral daylight (12 h light/12 h dark at 22°C) were syringe-infiltrated with bacteria at 10⁵ colony-forming units (cfu) ml⁻¹. Samples were collected at 0- and 3-day post-inoculation. Each replicate consisted of 2 leaf discs (surface area 0.385 cm²) from the infected leaves of various plants. Leaf discs were further ground in 500 µl infiltration media and homogenized in a shaker incubator at 30°C for 30 min 200 rpm. Bacterial titers were determined by plating serial dilutions of ground samples on King's *B* medium agar plates supplemented with 100 mg l⁻¹ Rifampicin and 25 mg l⁻¹ Kanamycin. The average number of cfu per cm² was calculated.

Log-transformed data were statistically analyzed. The data shown are collected from more than 10 technical replicates of two independent experiments.

4.4 Results and discussion

4.4.1 A subset of NPR1-dependent genes during systemic acquired resistance were up-regulated in *BOP1-oe*

Members from the NPR1 protein family interact with each other to form heterodimers, especially proteins within the NPR1-subclade that have significant roles in SA perception during SAR (Saleh et al., 2015; Castelló et al., 2018; Yan and Dong, 2014; Ding et al., 2018; Fu et al., 2012). These heterodimers could recruit ubiquitin ligases, regulate nuclear localization, or influence recruitment to DNA for the activation of target genes. A bimolecular fluorescence complementation experiment was carried out to confirm a report of Castello et al. (2018) showing that BOP1 and BOP2 can interact with NPR1 *in planta*. As shown in the Figure 4.1, reconstitution of yellow fluorescent protein (YFP) fluorescence in the nuclei of transformed *Nicotiana benthamiana* leaf cells indicates that BOP2 forms a complex with NPR1 in plant cells. A similar result was obtained for BOP1 (data not shown).

Transcriptome analysis of *BOP1-oe* versus wild-type plants by GO analysis found the enrichment of defense genes from various defense pathways (Chapter 3). Further RNA-seq experiments confirmed this finding and linked BOP1 and BOP2 with innate immunity. Systemic study of both microarray and RNA-seq indicated that a large number of genes up-regulated in *BOP1-oe* plants were classified as SA-related (Figure 3.1 and Supplemental Dataset 4). Since NPR1 is the major regulator in SA-mediated plant defense response, we asked if *BOP1-oe* plants rely on NPR1 to regulate defense machinery. A series of comparisons between up-regulated genes

in *BOP1-oe* and publicly available transcriptomic data for mutants of *NPR1* were carried out. Two sets of data were used for comparison: first, the early genomic response to SA of wild-type and *npr1-1* mutants (Blanco et al., 2009); second, the genome-wide reprogramming of *npr1-3* mutants after an SA-elicited SAR response (Shearer et al., 2012). Figure 4.2A shows that nearly half of NPR1-dependent SA-induced genes early in the defense response are also up-regulated in *BOP1-oe* plants. Among these 74 genes with NPR1-dependent induction during early SA perception (Blanco et al., 2009) were *CBP60g*, *CERK1*, and various *WRKY* transcription factors (*WRKY18*, -38, -46, and -60) (Supplemental Dataset 6). In addition, 193 NPR1-dependent genes late-induced during a SAR response were also up-regulated by overexpression of *BOP1* (Figure 4.2B). Some of these genes are considered as markers of both SAR and PTI, including *FRK1*, *NPR3*, *PR1*, *PHYTOALEXIN DEFICIENT 4*, *SARD1*, *SARD4*, and multiple *WRKY* transcription factor coding genes (*WRKY6*, -15, -18, -25, -33, -38, -40, -46, -58, -60, and -70). The fact that several key transcription factors under SA regulation (e.g. *CBP60g*, *SARD1* and *SARD4*) were induced in *BOP1-oe* plants indicates that BOP1/2 potentially regulates plant defense partially through an SA-dependent signaling pathway. NPR1 serves a central hub for co-ordinating SA-dependent defense responses. These observations prompted me to test if the defense mechanism of BOP1/2 relies on NPR1 for integration.

4.4.2 Inactivation of NPR1 modifies *BOP1-oe* plant architecture

BOP1 and BOP2 overexpression phenotypes are corrected by mutation of clade I TGA factors showing that BOP1/2 require these TGAs for function (Chapter 2). A similar approach was used to test if BOP1 requires NPR1 to exert changes in plant development. A null allele *npr1-3* (Cao et al., 1997) was introduced into the *BOP1-oe* mutant by crossing and the resulting plants were analyzed. With the inactivation of *NPR1*, a small but significant change in *BOP1-oe* plant height

and leaf size was observed (Figure 4.3A, C, and D). The *BOP1-oe npr1-3* plants remained short, but plant height was double compared to *BOP1-oe* plants (Figure 4.3C and D). By contrast, the rosette diameter of *BOP1-oe npr1-3* plants was smaller than *BOP1-oe* and the other genotypes tested (Figure 4.3A).

ROS produced in the plant cell wall serves a signaling molecule and directly as an oxidant involved in cell-wall cross-linking (O'Brien et al., 2012; Kärkönen and Kuchitsu, 2015; Mhamdi and Van Breusegem, 2018). In Chapter 3, a transient but enhanced ROS burst was detected in mature leaves of *BOP1-oe* after flg22 elicitation. Further examination ruled out that overexpression of *BOP1* can lead to an extended increase of ROS in young seedlings (Supplemental Figure S3.3A-C). Here, we tested the background ROS in mature leaves of WT, *BOP1-oe*, *npr1-3*, and *BOP1-oe npr1-3* under short-day conditions using older, soil-grown plants. Under these conditions, *BOP1-oe* plants showed stronger ROS staining at the leaf margin, especially at the lobes, compared to WT (Figure 4.3B). The leaf margin shape is controlled by a series of development regulators, such as CUP-SHAPED COTYLEDON and KNOTTED-LIKE FROM ARABIDOPSIS 2 and 6 (Bilsborough et al., 2011; Belles-Boix et al., 2006). All these genes are linked to boundary formation (Hepworth and Pautot, 2015). The enhanced ROS staining at the lobe of *BOP1-oe* leaves might alter growth through regulation of boundary genes.

4.4.3 BOP1/2 regulate target genes through an NPR1-independent pathway

Given that NPR1 contributes to pattern-triggered immunity (Ding et al., 2018; Liu et al., 2020) and approximately one-third of NPR1-dependent defense genes during early SA perception and SAR were up-regulated in *BOP1-oe* plants (Figure 4.2), it was intriguing to test if BOP1 confers plant immunity by NPR1-dependent mechanisms. To test if BOP1 regulates target genes in an

NPR1-dependent fashion, I tested several PTI-related genes that are known to be induced by overexpressing *BOP1*. The transcript levels of *WRKY18*, *WRKY38*, *WRKY60*, *WRKY62*, *SARD1*, and *CBP60g* were examined in *BOP1-oe*, *BOP1-oe npr1-3*, and wild-type seedlings. In agreement with our previous results, these candidate genes were significantly increased in *BOP1-oe* plants compared to wild-type (Figure 4.4). Inactivation of *NPR1* showed no significant effect on most of these genes except attenuated expression of *WRKY62* and *FLS2* (Figure 4.4). It's worth mentioning that during a SAR response, *WRKY18*, *WRKY38*, and *WRKY60* are induced through an NPR1-dependent mechanism. These WRKY transcription factors can be induced in the absence of NPR1 showing that BOP1 is sufficient to mount a defense response using some of the NPR1-regulated defense network. CBP60g and SARD1 are major transcription factors of SA biosynthesis during a PTI response (Wang et al., 2011; Sun et al., 2015, 2018). Both *SARD1* and *CBP60g* are direct target of BOP1 under flg22 elicitation (Figure 3.7E and F). The transcripts level of *SARD1* was not affected by inactivation of *NPR1* in *BOP1-oe*, but the expression of *CBP60g* was attenuated. Although both SARD1 and CBP60g are involved in SA biosynthesis during defense response, they regulate two parallel SA-biosynthesis pathways (Zhang et al., 2010). Constitutive expression of *SARD1* is sufficient to increase plant resistance to pathogens but not *CBP60g*, of which activity likely relies on Ca^{+} signals (Zhang et al., 2010). We then tested the PTI response of *BOP1-oe npr1-3* treated with *P. syringae hrcC*- mutant (*Pst hrcC*⁻). Results in Figure 4.5 showed that *BOP1-oe npr1-3* was not compromised in defense against *Pst hrcC*⁻. Collectively, these data suggest BOP1 can regulate a large part of NPR1-regulated defense mechanisms to mount a successful PTI response.

Figure 4.1 BOP2 can form a complex with NPR1 *in vivo*

The BiFC assay was employed to examine BOP2 interaction with NPR1 *in planta*. The N-termini of YFP (nYFP) or C-termini (cYFP) were cloned in-frame to make fusions at the C-terminus of BOP2, NPR1, or the TGA bZIP transcription factor PAN. Constructs were transiently expressed in 4-week-old tobacco leaves. Reconstitution of YFP fluorescence was examined by confocal microscopy. The adaxial surface of leaves was imaged using approximately 20 leaf discs prepared from 5 leaves per genotype. (A) 35S:YFP was used as a positive control for infiltration. Yellow fluorescence was detected in the cytoplasm and nucleus as expected. (B) BOP2-nYFP alone was used as a negative control. No fluorescence. (C) BOP2-nYFP + PAN-cYFP was used a positive control. Yellow fluorescence was observed in the nucleus and cytoplasm. (D) BOP2-nYFP + NPR1-cYFP. Yellow fluorescence was observed in the nucleus. Scale bar, 20 μ m.

Figure 4.2 A subset of NPR1-dependent defense genes was up-regulated in *BOP1-oe* seedlings

Venn diagrams, depicting the overlap between differentially expressed genes whose expression was altered in BOP1 overexpression mutant (*BOP1-oe*) seedlings and published microarray data on *npr1* loss-of-function mutant during early SA induction (A) and SAR response (B). (A) Venn diagram showing overlap between *BOP1-oe* up-regulated genes and candidate SA-induced genes that are NPR1-dependent (Blanco et al., 2009). (B) Venn diagram showing overlap between *BOP1-oe* up-regulated genes and SAR-inducible genes that are NPR1-dependent (Shearer et al., 2012).

Figure 4.3 Abolishing *NPR1* alters *BOP1-oe* phenotype

Plants homozygous for a *BOP1-oe* transgene were crossed to plants homozygous for an *npr1-3* mutation. (A) Top view of 4-week-old wild-type (WT), *BOP1-oe*, *npr1-3*, and *bop1-oe npr1-3* SD-grown plants. Scale bar, 1 cm. (B) Rosette leaves of SD-grown 6-week-old plants were stained with 3-3'-diaminobenzidine (DAB) for the detection of H_2O_2 . The number of plants with shown ROS levels is given at the top right of each image. Resting ROS levels in *BOP1-oe* and *BOP1-oe npr1* plants was higher than in wild-type and *npr1-3* plants in the majority of samples. Scale bar, 2 mm. (C) Representative *BOP1-oe* and *BOP1-oe npr1-3* plants are shown. Scale bar, 0.4 cm. (D) Quantitative analysis of plant height in populations of F2 plants for the genotypes indicated. Asterisk indicates a significant difference between *BOP1-oe* and *BOP1-oe npr1-3* genotypes ($n > 20$, Student's t-test, $P < 0.001$).

Figure 4.4 BOP1 promotion of PTI-related genes does not require NPR1

Upregulation of PTI-related defense genes in *BOP1-oe* transgenic plants was not abolished in *BOP1-oe npr1-3* seedlings. Seedlings were germinated on MS agar plate supplemented with 1% sucrose for ten-days under continuous light condition. Each replicate contains 10-15 seedlings. Transcript levels of *WRKY18*, *WRKY58*, *WRKY60*, *WRKY62*, *FLS2*, *RBOHD*, *SARD1*, and *CBP60g* were monitored in wild-type (WT), *BOP1-oe*, and *BOP1-oe npr1-3* plants using RT-qPCR. Data shown are the mean $\pm$ SE of three biological replicates. Lowercase letters represent significantly different groups ($p \leq 0.001$, one-way ANOVA with Tukey's post-hoc test).

Figure 4.5 BOP1 regulates PTI response independently from NPR1

PTI response is enhanced in *BOP1-oe* and *BOP1-oe npr1-3* mutant plants compared to the wild type (WT). Box plots of growth of *Pst hrcC-* in WT and mutant plants are shown. Leaves of four-week-old plants grown under neutral daylight conditions were syringe-infiltrated with bacteria at 10^5 colony forming units (CFU) ml^{-1} . Log-transformed data are means $\pm$ SD of replicates ($n > 10$) collected from two biological replicates. Each sample contains two leaf disks from separate plants. Means with different lowercase letters are significantly different ($p \leq 0.01$, one-way ANOVA with Tukey's post-hoc test).

CHAPTER 5

Conclusions and future directions

5.1 General conclusion

BTB-ankyrin proteins are a class of transcription co-factors present in the sequenced genomes of all land plants. In simple mosses, BTB-ankyrin proteins include both NPR1-like and BOP-like subtypes, indicating an origin that predates the emergence of land plants (Saleh et al. 2011). In both monocot and dicot species, BTB-ankyrin proteins regulate two of the most important functions in plants: development and defense. First discovered in *Arabidopsis*, the *BOP1* and *BOP2* genes examined in my thesis regulate a set of developmental traits important for crop yield: leaf architecture, branching architecture, and abscission. The manifestation of these traits is precisely regulated by growth patterns at junctions between organs and the plant body. Indeed, regulatory changes targeting maize BLADE-ON-PETIOLE1 led to an expanded expression in female reproductive shoots. This change had a critical role in transforming ancient teosinte into the domesticated corn that modern farmers grow in fields (Dong et al., 2017).

My thesis examines the mechanism of BLADE-ON-PETIOLE activity in development and defense using *Arabidopsis thaliana* as a study species. The major findings can be summarized as the following points:

1. Clade I TGA bZIP transcription factors are key functional partners of BOP1/2 in regulation of plant development

BTB-ankyrin proteins including BOP1 and BOP2 lack a DNA-binding domain and require other transcription factors for recruitment to DNA. Yeast-two hybrid studies identified TGA bZIP transcription factors as serving this role for NPR1. Before my thesis, the only known functional partner of BOP1/BOP2 was the clade V TGA8/PAN transcription factor required in flower

development (Hepworth et al., 2005). Here, genetics and biochemical experiments identified clade I TGA1/TGA4 transcription factors as broad functional partners of BOP1/2. Phenotypic analysis of loss-of-function mutant and expression studies showed that clade I TGAs function at boundaries with BOP1/2 to regulate meristem integrity, flowering, and inflorescence architecture. Biochemical studies showed that clade I TGA and BOP1/2 proteins form a complex and co-localize at bZIP-binding element-containing sites in the promoter of boundary gene *ATH1*, contributing to its activation. Our findings revealed a previously undiscovered developmental role for clade I TGAs.

2. A defense role for BOP1/2 involving clade I TGA1/4 in pattern-triggered immunity

BOP1 and BOP2 repression of growth relies on clade I TGA transcription factors. These TGAs also function in pattern-triggered immunity and SAR. Since clade I TGAs interact with NPR1 to regulate SAR, we hypothesized that BOP1 or BOP2 might interact with clade I TGAs for pattern-triggered immunity. To test this idea, microarray and RNA sequencing were used to examine the transcriptome of plants with loss or gain of BOP function compared to wild-type and *tga1 tga4* mutants. Compared to wild-type plants, nearly three thousand genes were up-regulated in *BOP1-oe* stem. One third of these genes were classified as “stimuli-related” indicating a potential role for BOP1/2 in plant responses to environmental stresses. A large portion of these gene clustered with pattern-triggered immunity where clade I TGA factors play a role. To further elucidate the underlying regulatory mechanism of BOP1/2, a large-scale transcriptomic study using next-generation RNA-sequencing was carried out using flg22 to trigger a PTI response. This analysis revealed a substantial shift in the transcriptome of *BOP1-oe* plants towards wild-type plants treated with flg22. We noticed that *bop1 bop2* and *tga1 tga4* mutants shared a large group of genes that

were insensitive to flg22 compared to the wild-type. Pathogen assays confirmed specific defect in pattern-triggered immunity suggesting that clade I TGAs and BOP1/2 co-regulate defense genes contributing to this response. Mechanistically, we found that BOP1 is recruited to the promoter of two clade I TGA-regulated genes but only under flg22 treatment. We also found that BOP1/2 and clade I TGA interact with a group of flg22-induced WRKY transcription factors. Several WRKY binding sites exist in the vicinity of bZIP binding motifs in the promoters of the two defense genes we tested. We hypothesize that interactions between all three classes of proteins might be required for specific activation of genes under defense conditions. Altogether, these results suggest that BOP1/2 regulate pattern-triggered immunity using functional partners similar to NPR1.

3. BOP1/2 regulate plant defense in a largely NPR1-independent manner

Clade I TGA regulation of plant immunity is substantially independent of NPR1 (Shearer et al., 2012; Wang and Fobert, 2013). Given that PTI activates both SA and JA/ET hormone signaling responses, we considered that BOP1/2 promotion of plant defense might rely on interactions with NPR1 to which it can bind. In Chapter 4, the phenotype of *BOP1-oe npr1-3* double mutants was examined. Inactivation of *NPR1* had a small but measurable impact on *BOP1-oe* morphology, but neither BOP1-regulated defense gene induction nor resistance to *P. syringae hrcC* was attenuated. Overall, we found that BOP1 can regulate PTI independently from NPR1.

5.2 Future directions

1. BOP1 and BOP2 interactions with TGA transcription factors

Although we have established a connection between BOP1/2 and clade I TGA1/4 in plant immunity, further bioinformatics analysis on the transcriptomic data we acquired might be fruitful

in pinpointing specific shared pathways. While clade I TGA factors play an important role in BOP1/2 dependent regulation of development, deletion of these bZIP genes causes only subtle boundary defects compared to *bop1 bop2* mutants. These defects are concentrated in leaves. Clade III and clade IV TGA transcription factor with roles in plant immunity are also expressed in boundaries. Future work will examine if these factors play a role with BOP1/2 in development or defense.

2. BOP1 and BOP2 interactions with WRKY transcription factors.

We found that BOP1 recruitment to the promoters of *SARDI* and CBP60g defense genes was specific to flg22-treated samples. Given that complexes between BOP1/2 and TGA1/4 form under resting conditions and TGA1 binding to the *SARDI* promoter is constitutive, WRKY transcription factors acting as repressors or co-activators might regulate the assembly of BOP1/2-containing activation complexes on the promoter. Several WRKY transcription factors (including WRKY31, WRKY60, and WRKY62) activated under flg22 treatment were found to form complexes with BOP1/2 and clade I TGA transcription factors. It is very important to decide if these WRKYs compete with clade I TGA1/4 to interact with BOP1/2, which would indicate a functional mode similar to NPR1-TGA3-WRKY70 (Saleh et al., 2015). Genetics study of these WRKY transcription factors using loss- and gain-of-function mutants might tell us how they influence the expression of BOP1/2 and clade I TGA co-regulated defense genes. Further biochemical assays using a luciferase reporter gene system can be employed to test if and how BOP1/2, TGA, and WRKY proteins working as a complex to influence the expression of *SARDI* (Hussain et al., 2018).

3. BOP1 and BOP2 targets in defense for CULLIN3-dependent ubiquitin ligases

Studies on animals have shown that BTB-ankyrin proteins functions as an anchor to connect CUL3 ubiquitin E3 ligase to target proteins, causing degradation (Godt et al., 1993). In Arabidopsis, this function has been discovered for NPR3 and NPR4 (Ding et al., 2018; Zhou and Zhang, 2020). Both NPR1-like proteins can serve as *bona fide* SA receptors to regulate NPR1 protein turnover via CUL3 ubiquitin E3 ligase-mediated degradation. Likewise, BOP1/2 serve as E3 ligase adaptors to regulate the abundance of PIF4 transcription factor during plant responses to the environment (Zhang et al., 2017) and the abundance of LEAFY transcription factor in promotion of flowering (Chahtane et al., 2018). Given BOP1/2 interact with various classes of proteins, including NPR1, TGA and WRKY transcription factors, it would be interesting to find out if BOP1/2 utilize ubiquitination as a mechanism to any of its interacting partner to regulate plant immunity.

In summary, my work shows that BOP1 and BOP2 with clade I TGA transcription factors regulate plant development and pattern-triggered immunity. My work also identified WRKY transcription factors an additional type of protein that interacts with BOP1/2 and clade I TGAs. This important work establishes a defense role for all members of the BTB-ankyrin family. The future challenge will be to understand how interactions between BTB-ankyrin members regulate transitions between the different layers of plant defense. Ultimately, a deeper understanding of plant defense systems might provide avenues for uncoupling defense trade-offs with growth in crop plants.

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