Large-scale studies and biophysical analysis of systems involved in plant immunity.

By

Kuai, Xiahezi, BSc.

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ABSTRACT

The field of plant immunity has progressed significantly in the last decade, driven primarily by both forward and reverse genetics and to a lesser extent by molecular biology techniques. However, many unknowns still remain before a more complete picture of this system can be achieved, which hinders our capacity to develop biotechnological solutions to ensure food safety for our growing population. Some of the problems that still need to be tackled relate to the multi-system involvement of some proteins, the interrelation of the different hormones, such as in trade-off systems, and the challenges of translating existing molecular knowledge into crop protection strategies.

The goal of this thesis was to develop new methods and to adapt existing ones to address the challenges and push the boundaries of our knowledge of plant immunity as a system. We have adapted ClueGO analyses to visualize functionally grouped Gene Ontology (GO) terms specific to Arabidopsis. We developed a transcription factor-coregulator identification strategy based on double-transcriptome analyses. Finally, we have adapted a biophysical method, differential scanning fluorimetry (DSF). We tested the usefulness of these methods by interrogating different immune proteins/genes of the model plant Arabidopsis thaliana.

Here is a summary of the major results obtained. In the realm of basal immunity, we discovered that clade I TGA transcription factors positively regulate this system by repressing WRKY transcription factors, which are negative regulators of the process. Furthermore, we have demonstrated that clade I TGA integrates into the growth-immunity trade-off system regulated by brassinosteroids by antagonizing the brassinosteroids-dependent suppression of basal immunity. In the realm of systemic
acquired resistance (SAR), we have demonstrated that clade I TGA recruits a specific novel glutaredoxin as a corepressor to dampen the expression of a set of SAR-regulated genes controlled by salicylic acid (SA) and the SAR-orchestrator, NPR1. Finally, we demonstrated that NPR1 binds SA and that this interaction leads to the destabilization of NPR1. More importantly, the method used to show the latter is scalable and can be used to develop novel chemistries capable of deploying plant immunity in the field.
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LIST OF ABBREVIATIONS

AHA - Azidohomoalanine  
AOS - allene oxide synthase  
AtCERK1 - Arabidopsis orthologue of OsCERK1  
ATH1 - arabidopsis thaliana homeobox gene1  
AzA - azelaic acid  
BAK1 - BRI1-associated receptor kinase 1  
BiFC - bimolecular fluorescence complementation  
BOP1/2 - blade-on-petiole1/2  
BRZ - brassinazole  
BTH – benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester  
BZR1 - brassinosteroid resistance 1  
CEBiP - chitin oligosaccharide elicitor-binding protein  
ChIP – chromatin immunoprecipitation  
CHX - cycloheximide  
co-IP - coimmunoprecipitation  
D35S - double 35S Cauliflower mosaic virus promoter  
DA - abietane diterpenoid  
DEGs - deferentially regulated genes  
DEX - dexamethasone  
DSC - differential scanning calorimetry  
DSF - differential scanning fluorimetry  
EF-Tu - elongation factor Tu  
EFR - EF-Tu receptor  
elf18 - the first 18 amino acids of EF-Tu  
ELISA - enzyme-linked immunosorbent assay  
ETI - effector-triggered immunity  
flg22 - a conserved stretch of 22 amino acids, located close to the N terminus of flagellin  
FLS2 - Flagellin Sensing 2  
FT - flowering locus t  
G3P - glycerol-3-phosphate  
GA – gibberellin  
GO - gene ontology  
HR- hypersensitive response  
IM - inflorescence meristem  
INA - 2,6-dichloroisonicotinic acid  
JA - jasmonic acid  
KNAT6 - knotted-like from arabidopsis thaliana 6
LRR - leucine-rich repeat
LRR-RK - leucine-rich repeat-receptor kinase
MAMPs - microbial-associated molecular patterns
MAPK - mitogen-activated protein kinase
MeSA - methyl salicylate
miR156 - microRNA156
NB - nucleotide binding
NLRs - NOD-like immune receptors
NPR1 - nonexpresser of pathogenesis related genes 1
OsCERK1 - rice chitin elicitor receptor kinase 1
PAMPs - pathogen-associated molecular patterns
PDGF-BB - homodimer of platelet-derived growth factor B-chain
PDGFRβ - Platelet-derived growth factor receptor β
PIF4 - phytochrome-interacting factor 4
PLA - proximity ligation assay
PNF - POUND-FOOLISH
PNY - PENNYWISE
PR1 - pathogenesis-related 1
PRRs - pattern recognition receptors
PSA - prostate specific antigen
*Psm ES4326 - Pseudomonas syringae pv. maculicola ES4326
Pst DC3000 - Pseudomonas syringae pv. Tomato DC3000
PTI - PAMP-triggered immunity
RCA - Rolling circle amplification
RKs - receptor kinases
RNA-seq - RNA-sequencing
ROS - reactive oxygen species
RPS4 - resistance to *Pseudomonas syringae* 4
RRS1-R - resistance to *Ralstonia solanacearum* 1
SA - salicylic acid
SAM - shoot apical meristem
SAR - systemic acquired resistance
SPL - squamosa promoter binding protein-like
*Tm* - Melting Temperature
Δ513 - C-terminal SA-binding domain of NPR1
CHAPTER 1 - INTRODUCTION

Crop protection is at the heart of our sustainable food, feed and fiber production. With a steadily growing human population, projected to reach 9 billion by 2050, food security is a global and vital challenge that must be addressed. Every year, crop yield is lost due to weed competition, insect herbivory and diseases caused by pathogens. Here in the developed world, we are privileged and are accustomed to having high crop yield and high crop quality in modern agriculture. These yield and quality traits are only possible through the combined use of improved cultivars, fertilizers and agrochemicals. In conventional agriculture, these chemicals include herbicides, insecticides, fungicides and antibiotics. There are other parts of the world, which are not so fortunate, and according to the FAO publication, The State of Food Insecurity in the World (2012), 870 million people worldwide are chronically undernourished, and most of them (850 million) live in developing countries. Agricultural growth is the key to reducing hunger and malnutrition. High crop quality and high crop yield will continue to be required to sustain our growing world population.

To add to the challenges faced by agriculture, regulatory bodies and consumers are demanding stricter regulatory safety margins, environmentally safer agrochemicals and practices, and low-cost goods, while at the same time the spectre of weed, pest and pathogen resistance to agrochemicals is mounting. As such, to ensure global food security, it is imperative to adopt a holistic approach to agriculture by not excluding or limiting the number of management tools at our disposal. These tools include biocontrol agents and chemicals that stimulate plant immunity. Studying the detailed molecular mechanisms of plant immunity can facilitate breeding technologies for the selection of
resistant crop cultivars and enable the rational design of agrochemicals that can enhance crop immunity.

Due to the importance of understanding the plant immune system for safeguarding agriculture productivity, substantial research efforts have been deployed to decode its molecular mechanisms. These efforts include genetic screenings, to discover important regulators, protein-protein interactions screenings, to decipher the relationships between key players, and RNA sequencing, to identify genes regulated by specific proteins or stimuli. Thanks to these efforts, we now have a good understanding of the plant immune system. However, despite the large amount of research being done, the number of methods used is rather limited. Repetitive and exhaustive uses of current methods have created a void in the field. New approaches are desperately needed to open new research directions and to translate the current molecular understanding of plant immunity into crop protection strategies.

1.1 Outline

The goal of the research presented in the thesis is to develop new methods and to adapt existing ones to study known immune regulators in depth, to discover unknown plant immune regulators and to advance the current molecular knowledge for translation into crop protection strategies.

Chapter 2 is a literature review that provides an overview of the current understanding of the plant immune system with an emphasis on systemic acquired resistance (SAR). A detailed review of the SAR signaling pathway, including metabolites and protein regulation, is reviewed in this chapter. In addition, the novel method developed and the existing ones adapted to study the plant immune system are
summarized in this chapter.

Chapter 3 is a manuscript in preparation, which uses large-scale transcriptome analyses to identify molecular switches controlling the plant immune system. In this study, transcriptome analyses of Arabidopsis thaliana (Arabidopsis) tga1tga4 mutant plants unraveled the role of transcription factors TGA1/4 in antagonizing brassinosteroids-mediated tradeoff between plant basal immunity and growth. Furthermore, a novel transcription factor-coregulator identification method based on transcriptome analyses was developed. Using this method, we have successfully identified a glutaredoxin, GRX480, as the coregulator of TGA1/4.

Chapter 4 is a published manuscript that develops target-based screening technology, which can be used to discover immunomodulating chemicals for crop protection. This chapter demonstrated, using differential scanning fluorimetry technology, that the receptor protein, nonexpresser of pathogenesis related genes 1 (NPR1), is destabilized after binding to salicylic acid. The scalable screening assay developed in this study can be used to translate molecular knowledge of the plant immune system into novel crop protection chemistries.

Chapter 5 is a published manuscript, which reveals that Arabidopsis thaliana (Arabidopsis) transcription factors PENNYWISE and POUND-FOOLISH fulfill the developmental transition from making leaves to the production of flowers by repressing lateral organ boundary genes, including genes encoding NPR family proteins BLADE-ON-PETIOLE1/2 (BOP1/2). Inactivation of BOP1/2 rescues flowering defect in pny pnf mutants. Moreover, BOP1 (NPR6) directly activates the transcription of ATH1, which encodes an important developmental and flowering transcription factor. Finally,
transcriptome analysis of transgenic plants overexpressing BOP1 revealed that BOP1 suppresses flowering process in part through the promotion of jasmonic acid biosynthesis.

Chapter 6 is a discussion of the entire thesis. An important step for translating the knowledge gained in the lab on the model plant Arabidopsis into crop protection in the agricultural field will be discussed in depth. Further research approaches will be also be discussed. Finally, conclusions for the entire thesis will be made.
CHAPTER 2 – LITERATURE REVIEW

2.1 Plant immune system

A compelling analogy for the plant immune system is the security system of a government facility or any other valuable entities worth protecting for this matter. What are the protective components involved in such a security system? First, there should be a secured building with a password-protected door to make sure enemies cannot just walk into the government facility freely. Next, internally, a surveillance system will be used to monitor any intruder who has already passed the first line of defense (the door) and intends on doing harm. Finally, once an intruder is recognized internally, a team of security guards will be assigned to neutralize the intruder and prevent any damage to the government facility.

Plants use an analogous defense system to guard themselves against their enemies. In this thesis, we will focus on the plant defense system against fungal, bacterial and viral pathogens. To protect themselves, plants establish mechanical barriers, such as, cell walls, waxy epidermal cuticles and bark to shut pathogens out (Freeman and Beattie, 2008). Next, if a pathogen successfully passed the first line of defense, at the cellular level, different kinds of surveillance-type receptors are used as security cameras to monitor pathogen attacks (Dodds and Rathjen, 2010). These receptors are capable of binding to specific foreign molecules and triggering corresponding signaling cascades inside the plant cells. Finally, once a pathogen infection is perceived and defense signaling is initiated, plants will establish a series of defense responses to protect themselves against the invading pathogen. Such defense responses include the productions of toxic chemicals and pathogen-degrading enzymes to fight the pathogen,
and the deliberate cell suicide to prevent the spread of the infection (Muthamilarasan et al., 2013).

The mechanical barriers and pre-formed antimicrobial chemicals form the first line of plant defense. These are also known as the constitutive defenses since the barriers guard the safety of plants whether or not there is a pathogen intrusion. In contrast, the activation of surveillance-type receptors and the resistance responses are triggered by the invasion of pathogens. Therefore, the surveillance and the resistance systems constitute the inducible defense of plants. The primary focus of this thesis is to study the inducible defense system, which detects the pathogen invasion and initiates effective plant resistance that fights off the infection. In the first part of this literature review, we will view the plant immune system from the following two aspects: first, the surveillance mechanism which is constituted by a double-branched plant pathogen perception system; second, the resistance responses, including basal immunity and systemic acquired resistance.

2.1.1 Surveillance: a double-branched plant pathogen perception system

There are two different kinds of receptors used by plants to recognize pathogen attacks: the first type of receptors sense pathogen molecules outside of plant cells and the other type recognizes intracellular foreign molecules (Dodds and Rathjen, 2010). These two kinds of receptors are coined pattern recognition receptors (PRRs) and resistance (R) proteins respectively.

2.1.1.1 Extracellular surveillance: PRRs and PAMPs

Pattern recognition receptors (PRRs) are cell surfaced immune receptors, which can perceive pathogen molecules termed pathogen-associated molecular patterns
PAMPs) (Jones and Dangl, 2006).

PRRs are either receptor kinases (RKs) or receptor-like proteins (Zipfel, 2014). RK contains an extracellular ligand-binding domain, a transmembrane domain and an intracellular kinase domain, which functions as internal signaling domain. A receptor-like protein processes a similar structure as RK, but without the intracellular kinase domain. All PRRs penetrate cell membranes through their transmembrane domains and bind with pathogen molecules with their extracellular ligand-binding domains. The perception of PAMP can trigger structural changes in PRR (Bigeard et al., 2015). For a RK, this structural change activates its kinase activity to initiate defense signaling. In the case of receptor-like proteins that lack a kinase domain, interaction with another protein is required to trigger the intracellular signaling cascades. Plant genomes encode large numbers of RKs and receptor-like proteins that may serve as potential PRRs. For example, the Arabidopsis genome encodes 610 RKs and 57 receptor-like proteins (Dodds and Rathjen, 2010). Most of them have unknown functions.

A PAMP is a molecule associated with a group of pathogens, which often plays an important role in pathogen fitness or survival (Zipfel, 2014). Diverse types of molecules can serve as PAMPs, such as, the fungal polymer, chitin, the bacterial protein, flagellin and bacterial DNA (Yakushiji et al., 2009). The term, microbial-associated molecular patterns (MAMPs), is proposed, because such patterns also occur in nonpathogenic microorganisms (Bittel and Robatzek, 2007). In plants, the terms PAMP and MAMP are interchangeable.

The best-understood perception mechanism of PAMP by PRR is the recognition of the bacterial PAMP, flagellin by the Arabidopsis RK, Flagellin Sensing 2 (FLS2)
The protein flagellin is the principal component of the bacterial flagellum, a lash-like appendage used for motility. FLS2 is a leucine-rich repeat-receptor kinase (LRR-RK) that binds to a conserved stretch of 22 amino acids (flg22), located close to the N-terminus of flagellin (Chinchilla et al., 2006). Treatments with flg22 alone can be recognized by the plant FLS2 and induce the flagellin-triggered immunity. Therefore, experimentally, flg22 is often used as a proxy to flagellin. In addition to FLS2, BRI1-associated receptor kinase 1 (BAK1) is also involved in the flg22 perception. BAK1 was identified, as a positive regulator of the flg22 intracellular signal transduction, through a genetic screening (Chinchilla et al., 2007). bak1 mutant plants show normal flagellin binding but abnormal flagellin-triggered responses. Co-immunoprecipitation assays showed that flg22 induces a rapid interaction between FLS2 and BAK1. A crystal structure study of FLS2 and BAK1 ectodomains complexed with flg22 revealed more insights about this multicomponent complex (Sun et al., 2013). Besides directly interacting with FLS2, BAK1 acts as a co-receptor by recognizing the C-terminal side of the FLS2-bound flg22. Collectively, BAK1 is not only required for flg22-triggered signaling but is also involved in the perception of the PAMP complexed with FLS2. Interestingly, BAK1 is also the co-receptor required for brassinosteroid (BR) phytohormones perception and signaling. In the case of BR perception, BAK1 forms a complex with the BR receptor, BRI1. The receptor complex subsequently initiates the BR signaling (Li et al., 2002). The use of the common component, BAK1, in both flg22 and BR perceptions, would suggest a tradeoff mechanism through the limiting availability of BAK1. However, co-immunoprecipitation experiments have shown that the BR perception in Arabidopsis does not affect the association and the activity of the FLS2-
Thus, BAK1 is not a rate-limiting factor between flg22- and BR-triggered pathways.

In addition to flg22, BAK1 also plays a role in the perception of another bacterial PAMP, the elongation factor Tu (EF-Tu) (Chinchilla et al., 2007). EF-Tu is one of the most abundant proteins in prokaryotes (Weijland et al., 1992). It catalyzes the binding of an aminoacyl-tRNA to the ribosome (Weijland et al., 1992). The function of EF-Tu is crucial to the polypeptide elongation process of protein synthesis. *Arabidopsis* receptor kinase, EF-Tu receptor (EFR) perceives EF-Tu through direct binding to the first 18 amino acids of EF-Tu (elf18) (Zipfel et al., 2006). EFR was also shown to interact with BAK1 in a ligand dependent manner in vivo (Roux et al., 2011).

Another well-studied, classical PAMP is chitin. Chitin is a major constituent of fungal cell walls. The first discovered plant chitin-binding PRR is chitin oligosaccharide elicitor-binding protein (CEBiP) (Kaku et al., 2005). It was isolated from the plasma membrane of suspension cultured rice cells (Kaku et al., 2005). CEBiP is a receptor-like protein, which does not contain an obvious intracellular signaling domain. It requires the rice chitin elicitor receptor kinase (OsCERK1) to assist in intracellular chitin signaling (Shimizu et al., 2010). Structurally speaking, binding of a chitin octamer first induces the homodimerization of CEBiP and then a CEBiP homodimer forms a heterooligomeric complex with two monomers of OsCERK1. This series of structural changes result in a sandwich-type receptor system for chitin perception (Hayafune et al., 2013). Interestingly, different from the chitin perception in rice, the *Arabidopsis* orthologue of OsCERK1, AtCERK1, directly binds octamers of chitin. The binding event leads to homodimerization of AtCERK1, which subsequently activate an intracellular immune
signaling (Liu et al., 2012).

2.1.1.2 Intracellular surveillance: R proteins and effectors

The second types of receptors that can recognize specific intracellular pathogen molecules are R proteins. Each R protein is responsible for the specific perception of one effector (Jones and Dangl, 2006).

R proteins are also known as NB-LRR proteins. The name, NB-LRR comes from the domain structure of this family of proteins, which contain a nucleotide binding (NB) site and leucine-rich repeat (LRR) domains (Chisholm et al., 2006). The domain functions of R proteins are unclear. LRR domains have been implicated in effector interaction activities (DeYoung and Innes, 2006). However, LRR domains of the studied R proteins are generally conserved, which indicates LRR domains alone cannot account for the specificity of R protein - effector interactions. Therefore, the part of the R protein that determines the binding specificity to an effector is not clear.

Effectors are pathogen-secreted proteins that are directly injected into host cells to aid infection (Chisholm et al., 2006). Bacteria use a needle like structure, called the type three secretion system to inject effectors into plant cells (Hueck, 1998). Fungal effectors are delivered into host cells through an unknown mechanism. Since most biotrophic fungi form a specialized infection structure, the haustorium, it was hypothesized that fungi use haustoriums to deliver effectors into the plant intracellular space (Dodds and Rathjen, 2010). Unlike PAMPs, which are molecules involved in fundamental physiological functions of pathogen, effectors are produced by pathogens to exclusively aid with their infection. Effectors promote pathogenicity by repressing host immunity or altering host physiology to sustain pathogen growth. Most effectors have enzymatic activities aimed at
modifying host targets (DeYoung and Innes, 2006). Some of the effectors directly possess transcription factor activities in order to control host gene transcription (Kay et al., 2007).

The difference in the binding of foreign molecules determines the difference in the detection spectrums that PRR and R protein possess. PAMP is associated with not just one particular pathogen but with a group of pathogens, which means that a PRR is capable of sensing a group of pathogens. In comparison, each effector is very specific to one particular pathogen. Therefore each R protein can only perceive one pathogen (DeYoung and Innes, 2006).

Studies on the binding mechanism of R proteins and effectors have revealed intriguing and diverse modes of perception. R proteins generally perceive effectors through direct or indirect recognition (Chisholm et al., 2006). Direct mechanisms involve physical association of R proteins with effectors. Indirect recognition is dependent on accessory proteins. These accessory proteins can be effector-targeted host proteins or “decoy” proteins, which mimic the structure of a targeted host protein. Effectors modify accessory proteins to aid with infection. R proteins can detect changes in the accessory protein. The modification of an accessory protein by an effector can lead to the interaction of a R protein with an accessory protein or the dissociation of a pre-formed R protein–accessory protein complex. Such effector-triggered changes on the interaction state of an R protein with an accessory protein will subsequently initiate immune signaling events. Most recently, a new mode of R protein perception, integrated decoy mechanism was discovered (Roux et al., 2015). This mechanism appears to be a hybrid of the direct and decoy models. In such a mechanism, a decoy domain is incorporated into
an R protein. Binding of an effector to the decoy domain of an R protein can activate the receptor and immunity (Jones et al., 2016). The integrated decoy mechanism was first demonstrated by the recognition of a bacterial effector PopP2 through an *Arabidopsis* receptor pair (Roux et al., 2015). PopP2 possesses acetyltransferase activity. It acetylates multiple WRKY transcription factors. WRKY acetylation by PopP2 causes the loss of WRKY-DNA binding activities and the inactivation of immune genes. The *Arabidopsis* NB-LRR proteins RRS1-R (resistance to *Ralstonia solanacearum* 1) and RPS4 (resistance to *Pseudomonas syringae* 4) form a receptor pair. RRS1-R contains a WRKY transcription factor domain that binds DNA. Acetylation of the transcriptional decoy domain of the RRS1-R/RPS4 receptor complex disrupts the DNA binding, triggering the activation of the receptor complex and the induction of immune responses. How exactly the activated receptor complex initiates defense signaling is unknown. However, this integrated decoy mechanism with a decoy domain in a receptor pair turns the disruption triggered by the effector into a immunity booster and is a fascinating evolutional defense strategy.

Although the functions of effectors and their binding to R proteins are intensively studied, it is not known how R proteins then initiate immune signaling. Plants R proteins share sequence similarity with the animal immune receptors, NOD-like immune receptors (NLRs). Upon interactions with targeted molecules, NLRs are activated by self-oligomerization through their NB domains (Jones et al., 2016). The oligomerized NLRs form a platform to initiate immunity-activating signaling. It is possible that plant R proteins use a similar mechanism to initiate defense signaling.
2.1.2 Resistance

Plant disease resistance can be reviewed as a sum of PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI and ETI were first proposed and studied as two separated immune responses (Jones and Dangl, 2006). PTI was first thought to be a weak variant of ETI. ETI was defined as a stronger and faster defense mechanism. Hypersensitive response (HR) and SAR were exclusively restricted as parts of ETI, but not PTI. However, recent studies have blurred the differences between PTI and ETI. First, in general, PTI and ETI trigger very similar immune responses (Thomma et al., 2011). Second, PAMP molecules are sometimes found to initiate stronger responses than effectors (Wei et al., 1992). Conversely, several weak ETI responses have been reported (Wirthmueller et al., 2007). Last, both PAMPs and effectors have been shown to induce HR and SAR (Mishina and Zeier, 2007). Therefore, PTI and ETI are collectively reviewed under the umbrella term, plant disease resistance, in this thesis. Plant disease resistance includes basal immunity and systemic acquired resistance, which are reviewed below.

2.1.2.1 Basal immunity

Basal immunity is also known as basal immune response or basal resistance (Dodds and Rathjen, 2010). Defense responses involved in basal immunity are deployed at the site of the infection. Such responses include stomata closure to prevent pathogen entry, the production of reactive oxygen species (ROS), which have direct antimicrobial activities, the production of toxic phytochemicals such as, camalexin in Arabidopsis, reprogramming of immune gene expression and localized cell death (HR) to avoid the spread of the infection (Sawinski et al., 2013; Baker and Orlandi, 1995). Naturally, basal
immunity is triggered by the perception of PAMP or effector. Experimentally, basal immunity can be quantified by measuring ROS production at the infected leaves after a PAMP treatment, such as flg22 (Lozano-Durán et al., 2013).

The immediate downstream signaling event of the PAMP or effector perception is not clear, but Ca\(^{2+}\) is likely to be an immune signaling molecule. An influx of extracellular Ca\(^{2+}\) in the cytosol (Ca\(^{2+}\) burst) has been shown to occur 30s to 2 min after the PAMP perception (Nomura et al., 2012). In addition to Ca\(^{2+}\), ROS have also been implicated in defense signaling (Baker and Orlandi, 1995). However, the detailed role of Ca\(^{2+}\) or ROS is not known. One known signaling pathway involved in basal immunity is mitogen-activated protein kinase (MAPK) signaling (Bigeard et al., 2015). MAPK signaling is not directly activated by PRR or R protein. However, it is crucial to both PAMP and effector induced basal immunity. Downstream targets of MAPK include WRKY transcription factors, WRKY7, 8, 9, 11 (Adachi et al., 2015).

2.1.2.1.1 Transcriptional reprogramming mediated by WRKYs

WRKY transcription factors have been shown to play important roles by controlling transcriptional reprogramming involved in basal immunity. In plants, WRKY family contains a large number of members (74 members in Arabidopsis, 104 members in rice) (Pandey and Somssich, 2009). Many members of the WRKY family are known to be positive regulators of basal immunity while many others are found to negatively regulate the same process.

Various genetic studies in Arabidopsis demonstrated the positive roles that many WRKYs play in basal immunity. A gain-of-function study showed that the transient expression of WRKY29 in Arabidopsis leads to reduced disease symptoms after
*Pseudomonas syringae* infection (Asai et al., 2002). Loss-of-function studies revealed that several positive WRKYs play redundant roles in basal immunity. Effector (AVR3a)-triggered ROS production is significantly reduced in *WRKY7, 8, 9, 11* silenced tobacco plants, but not in the single *WRKY* silenced lines (Adachi et al., 2015).

Many reports using similar genetic tools have also demonstrated the negative regulation of WRKYs on basal immunity. Xu et al showed that the *wrky18 wrky40 wrky60* triple mutant was significantly more resistant to *Pseudomonas syringae* infection than wild-type plants, indicating that WRKY 18, 40, 60 are immune suppressors. WRKY48 was shown to be a negative regulator of basal immunity in experiments with a T-DNA insertion mutant and with gain-of-function transgenic plants (Xing et al., 2008).

It is rather intriguing that WRKYs exercise both positive and negative transcriptional control of basal immunity. Positive control can lead to the timely activation of basal immunity while negative control may build stronger immunity by repressing negative WRKY regulators (as concluded in Chapter 3). Negative WRKY regulators can also interact with plant growth/development regulators to modulate immune responses. In order to allocate precious resources to growth, the brassinosteroid phytohormones induce the expression of immune-suppressing WRKY 15, 48 through the transcription factor BZR1 (Lozano-Durán et al., 2013). Furthermore, BZR1 also physically associate with WRKY40 to inhibit the expression of immune genes (Lozano-Durán et al., 2013).

### 2.1.2.2 Systemic acquired resistance

The perception of PAMP or effector can also trigger systemic acquired resistance (SAR). SAR is a broad-spectrum and ‘whole-plant’ resistance response (Vlot et al. 2009).
The term ‘broad-spectrum’ indicates that once induced, SAR will give rise to immunity against viral, bacterial and fungal pathogens. The term ‘systemic’ or ‘whole-plant’ relates to the fact that once the immune response is triggered in any part of the plant, immunity will be deployed throughout the plant making SAR the most agronomically relevant type of plant immunity. Two other features of SAR are that it is characterized by PR genes induction and also its deployment requires the build-up or at least the presence of endogenous salicylic acid (SA) (Vlot et al. 2009). PR genes encode Pathogenesis-Related (PR) proteins. Some of the PR proteins have direct anti-microbial functions and the others are involved in strengthening the cell walls or the production of toxic chemicals (Kauffmann et al., 1987).

SAR is naturally triggered by perceptions of PAMPs or effectors. Experimentally, SAR can be induced by SAR-inducing pathogen, named avirulent pathogen. The first avirulent strain, Pseudomonas syringae MM1065 was discovered through a screen of phytopathogenic Pseudomonas strains (Dong et al., 1991). A strain was classified as virulent if it elicited disease phenotypes in Arabidopsis. Conversely, a strain is classified as avirulent if it induced plant resistance responses. Genetic studies showed that avirulence of strain MM1065 is attributed to a specific avr gene, avrRpt2 (Whalen et al., 1991). Later studies revealed that avrRpt2 encodes a bacterial type III effector protein, which can be sensed by a plant surveillance-type intracellular receptor R protein RPS2 (Mackey et al., 2003). RPS2 subsequently induces resistance responses against P. syringae expressing the type III effector gene avrRpt2. SAR can also be chemically triggered by systemically treating a plant with SA or other chemical inducers, such as 2,6-dichloroisonicotinic acid (INA) (Vernooij et al. 1995) and benzo-(1,2,3)-

### 2.2 SAR signaling pathway

The SAR signal transduction process involves the perception of PAMP or effector in one part of the plant. Such perception subsequently triggers the production of an unknown mobile signal locally (Dempsey and Klessig, 2012). This unknown mobile signal is translocated throughout the plant and induces the accumulation of SA. The stress-induced production of SA involves the plastid associated isochorismate pathway where isochorismate synthase converts chorismic acid to isochrismate that is further modified to salicylate by an uncharacterized pathway (Dempsey et al., 2011). The accumulation of SA can induce *PR* genes systemically (Vlot et al. 2009).

In some plants, such as the model plant *Arabidopsis*, SA concentrations rise from a basal level to a high level during the deployment of SAR (Vlot et al. 2009). In others, such as rice, the basal level of SA is already high and does not rise significantly during the SAR deployment (Vlot et al. 2009). Nevertheless, despite the absence of an increase in SA concentrations in rice, SA is required for the SAR deployment.

#### 2.2.1 SAR mobile signal

Because SAR is an immune response that is first triggered at a local part of a plant and then gives rise to resistance of the whole plant, ensuing timing and precise signal transduction from the local infected part to the systemic tissues is a critical step to the deployment of SAR. Due to the importance of the mobile signal to plant immunity, the identification of such agent has been the focus of many labs. In theory, the mobile signal
that is capable of transducing the immune message should fulfill the following four attributes. First, the signal should accumulate locally in response to SAR-inducing pathogen infection. The first attribute is not an absolute requirement but rather an important aspect to test experimentally. Because instead of increasing its concentration after the pathogen attack, the signal could be present in plants at high level and not rise, but translocate systemically in response to the SAR induction. Second, the mobile signal should be able to move systemically. Third, mutating the biosynthesis of this signal should abolish SAR in plants. Finally, local application of such agent will induce resistance of the whole plant.

One obvious candidate to be the SAR mobile signal is SA. This is because SA accumulates both locally and systemically after pathogen attack (Ryals et al., 1996). However, this hypothesis was dismissed by a study published in 1994. Vernooij et al constructed grafted tobacco plants containing rootstocks and scions derived from wild type (WT) or SA-deficient plants. After SAR induction in the SA-deficient rootstock, SAR fully established in the chimeric plants consisting of WT scions and SA-deficient rootstocks. This result indicated that although the infected SA-deficient rootstock was incapable of producing SA, an unknown mobile signal was still produced and induced the resistance in the distal scions of the chimeric plants. Therefore, SA is not the mobile signal of SAR.

The second chemical that was proposed as the SAR mobile signal is jasmonic acid (JA) (Truman et al., 2007). The authors presented several lines of evidence to support the conclusion. First, JA accumulated in phloem exudates of Arabidopsis leaves challenged with SAR-inducing pathogen. Moreover, SAR was induced after JA application and
abolished in *Arabidopsis* mutant impaired in JA biosynthesis, *opr3*. However, their conclusion was challenged by another study published two years later because of issues with the experiments reproducibility. Attaran et al, 2009 showed that SAR response were intact in *Arabidopsis* JA biosynthesis mutants, *opr3* and *dde2*.

In 2008, methyl salicylate (MeSA) was suggested as the mobile signal of SAR (Park et al., 2008). The authors proposed a model in which SA is first converted to MeSA by SA methyl transferase in local tissue after pathogen challenge, and then MeSA, as the mobile signal, would move throughout the plant. Finally, in distal tissues, a MeSA esterase would convert MeSA back to SA, which subsequently induces the resistance. Their model was supported by SAR induction experiments using grafted tobacco plants containing rootstocks and scions derived from wild type (WT) or MeSA esterase-silenced plants. Challenging WT rootstock could not induce SAR in MeSA esterase-silenced scion, indicating the importance of MeSA esterase to the induction of SAR at the distal tissues. Similar experiments also demonstrated the importance of SA methyl transferase in SAR. Inducing SAR in local tissues that are incapable of converting SA to MeSA could not stimulate SAR response in WT distal tissues. However, contrary to their data, MeSA does not appear to be the mobile signal in *Arabidopsis*. The *Arabidopsis* MeSA deficient mutant, *bsmt1*, was intact in mounting SAR (Attaran et al., 2009). This result raised questions about the validity of MeSA as the general plant mobile signal for SAR.

In 2009, Jung et al identified azelaic acid (AzA) using an elegant comparative method. It was shown previously that vascular sap produced by the leaves infected with SAR-inducing bacteria, called petiole exudate, confers disease resistance to previously unexposed plants (Maldonado et al., 2002). The authors scanned for small molecules that
are enriched in the active (SAR-induced) but not in inactive (mock-induced) exudates using gas chromatography coupled with mass spectrometry (GC-MS). A dicarboxylic acid AzA was identified. Initial tests showed that AzA is a promising candidate for SAR mobile signal. Local application of AzA could induce SAR in distal tissues and radioactive feeding experiment indicated that AzA could move systemically in plants as locally injected AzA can be detected in the distal tissue. However, other experiments questioned the capacity of AzA as the SAR mobile signal. SA concentrations were not changed in AzA-treated plants. More importantly, microarray analysis of AzA-treated plants showed that AzA does not alter defense-related gene transcriptions. It appears to be that instead of inducing defense response directly, AzA functions as a primer for plants to mount faster or/and stronger immune responses.

An important component of carbohydrate and lipid metabolism processes, glycerol-3-phosphate (G3P), was proposed as the mobile inducer of SAR in 2011. Chanda et al demonstrated that G3P accumulates both locally and systemically after local pathogen infection. Moreover, mutants that are deficient in G3P biosynthesis could not establish SAR after induction. However, local application of G3P did not confer resistance to the systemic tissues. Therefore, G3P appears to be an important but not sufficient mobile signal for SAR.

The most recent chemical that was proposed to be the SAR mobile signal is abietane diterpenoid (DA) (Chaturvedi et al., 2012). Just like AzA, DA was purified as a SAR-activating agent from the vascular sap of Arabidopsis leaves treated with a SAR-inducing microbe. Feeding experiments with radioactive DA showed that DA could move systemically in plants. Furthermore, resistance of the whole plant can be triggered by
local application of DA. DA is shown to be a potent candidate for the SAR mobile signal. However, the role of DA cannot be fully determined due to the poor understanding of DA metabolic processes in plants.

To date, despite many strong attempts, the mobile signal of SAR still remains unclear.

2.2.2 SA signaling

Salicylic acid (SA) is an endogenous plant hormone essential to the deployment of a long-lasting, broad-based immunity, SAR. SA protects plants from a wide range of phytopathogens by mediating immune response at both local and systemic level (Vlot et al., 2009). SA has also been found to participate in abiotic stress responses. For instance, exogenous SA applications induce tolerance to copper toxicity (Mostofa and Fujita, 2013). In addition to its role in biotic and abiotic stress resistances, SA can influence plant flowering and thermogenesis (Vlot et al., 2009). Due to its biological significance, the signal transduction of SA has been intensely studied. Still, not much is known about the molecular details of the SA signaling pathway and the SA receptor remained unidentified for decades.

In 2012, two independent groups contributed new insights into the SA-perception and signaling-cascade. Interestingly, these advances are all centered on the NPR1 protein. One study showed that NPR1 can directly bind SA and acts as an SA-receptor (Wu et al., 2012). The other group proposed that two NPR1 paralogs, NPR3, and NPR4, bind SA and control the proteasome-mediated degradation of NPR1 through their interaction with NPR1 (Fu et al., 2012). Both groups however, demonstrated the indispensable role of NPR1 in SA signaling.
2.2.2.1 NPR1, the receptor of SA

NPR1 is a central regulator of plant immunity, which controls both local resistance and SAR. Plants lacking a functional NPR1 protein are unable to undergo SAR or express the SAR-marker gene \textit{PR1}, and as a result succumb to biotrophic pathogenic challenges (Cao et al., 1994; Delaney et al., 1995). Later, it was shown that NPR1 is a transcription coactivator (Rochon et al., 2006). The molecular mechanisms of NPR1 function are best understood in the case of \textit{PR1}. Transcription of \textit{PR1} is repressed by TGA2 transcription factor under SA concentration existing in naïve cells (Zhang et al., 2003; Rochon et al., 2006). Upon build-up of SA, NPR1 activates \textit{PR1} transcription by forming an enhansome with TGA2 on the promoter and negating the repressor activity of TGA2 (Boyle et al., 2009). The formation of the enhanceosome is well understood. However, the exact role played by SA leading to its formation remains unclear until 2012.

A novel and interesting feature of NPR1, aside from being a newly discovered and important phytohormone-receptor, is the requirement of the transition metal copper for SA-binding. Mutation of Cys521 and Cys529 of the C-terminal transactivation domain not only disrupts the SA-binding capacity of NPR1, but also eliminates the recruitment of copper by NPR1 (Wu et al., 2012). This is the first plant example of a copper-binding protein acting as a transcription regulator. The fact that NPR1 is a metalloprotein explains why it took so long to identify it as an SA receptor. Many researchers, by default, include EDTA as a chelator when preparing buffers. However, the recruitment of SA by NPR1 is EDTA-sensitive and its presence in buffers precludes SA from binding to NPR1 (Wu et al., 2012). Despite the fact that NPR1 is the first
copper-binding transcription-regulator discovered in plant, it is not the first time that copper is found to play a critical function in hormone signal-transduction pathway. The high-affinity binding-activity of the gaseous plant hormone, ethylene, to the ethylene receptor, ETR1, also requires copper as a cofactor (Rodriguez et al., 1999). As is the case of SA in NPR1, ethylene is coordinated to copper in the ETR1 hormone-binding pocket.

2.2.2.2 Mechanistic description of SA perception by NPR1 and PR1 activation, feature TGA2/5/6

Mechanistically, after perception of SA by NPR1, the NPR1 protein will ‘open up’ and release the C-terminal transactivation domain from the auto-inhibition by the N-terminal BTB/POZ domain (Rochon et al. 2006; Wu et al. 2012). NPR1 does not contain a DNA-binding domain and requires interaction with a DNA-binding protein for its recruitment to a promoter. As such, NPR1 is not a transcription factor, but a transcription coactivator. Recruitment to the SAR marker gene PRI is effected by the TGA2-clade of transcription factors and occurs in vivo only after perception by SA (Subramaniam et al. 2001; Zhang et al. 2003). In vitro, NPR1 can interact with TGA2 without SA (Boyle et al. 2009). This observation may be due to the polydispersity of NPR1 protein preparations, in which some of the NPR1 molecules could be in an ‘open’ state. NPR1 contains two protein-protein interaction domains of which we are aware. These are the BTB/POZ domain in the N-terminus (Aravind & Koonin 1999) and the ankyrin repeats located in the central portion of the protein (Cao et al. 1997; Ryals et al. 1997). The ankyrin repeats are the domain responsible for the stable formation of the TGA/NPR1 complex. However, the BTB/POZ domain also contacts the TGA factors, and specifically in the case of TGA2, the domain interacts with the N-terminus of TGA2 (Boyle et al. 2009).
TGA2 is recruited to *PRI* both pre- and post-SA treatment and at its core, TGA2 is a transcriptional repressor. The repression domain is located in the N-terminus of TGA2. The function of the TGA2 repression domain is negated by the binding of the BTB/POZ domain of NPR1. Indeed, when one deletes the repression domain from TGA2, the NPR1 BTB/POZ becomes obsolete with respect to *PRI* induction and can be deleted from the protein without consequences (Boyle et al. 2009).

### 2.3 The roles of other NPR family proteins in plant immune system

In the model system *Arabidopsis*, NPR1 is part of a six-member multigene family (NPR1 through 6). The function of NPR2 remains elusive. NPR3 and NPR4 have been proposed to be SA-receptor with a role in the degradation of NPR1 (Fu et al., 2012). However, immunity functions in the absence of NPR3 and NPR4. NPR5 and NPR6 play roles in flower development (Hepworth et al., 2005). NPR1 is the one and only NPR-family member required for the deployment of immunity.

#### 2.3.1 NPR3/NPR4 and plant immunity

NPR1 is a positive regulator of SAR. Recently, additional members of the NPR family, NPR3 and NPR4, were shown to negatively regulate SAR (Liu et al., 2005; Zhang et al., 2006; Fu et al., 2012). Analysis of conceptual gene products revealed that NPR3 and NPR4 respectively share 34.5 and 36.0% amino acid-conservation with NPR1, specifically in the BTB/POZ and ankyrin repeat domains (Liu et al., 2005). Protein alignments indicate that all three NPR share four (4) conserved cysteines in their BTB/POZ domain, and a stretch of five (5) variable basic-amino acids at the C-terminal, that may be involved in nuclear localization (Shi et al., 2013). The structural similarities among these three protein appears to extend to their functional roles including SA-
perception and interaction with members of the TGA family of transcription factors (Després et al., 2000; Rochon et al., 2006; Kinkema et al., 2000; Subramaniam et al., 2001; Fan and Dong, 2002; Mou et al., 2003).

At the organ level, expression of NPR1/NPR3/NPR4 appears to occur in different locations. Promoter-driven GFP expression observed with fluorescence stereomicroscopy, demonstrated that NPR1 was detectable only in leaves, NPR4 only in mature siliques and roots, while NPR3 was expressed in relatively high quantities in the young flower (Shi et al., 2013). At the subcellular level, NPR3/NPR4-TGA2 interactions have been observed primarily in the nucleus, when studied in onion epidermal cells and Arabidopsis mesophyll protoplasts (Zhang et al., 2006). While nuclear localization of NPR1 has been shown definitively, differing reports have suggested that NPR1 can also be observed in the cytoplasm as well (Després et al., 2000).

The pathology surrounding npr1/npr3/npr4 mutants has displayed different phenotypes under the exact and differential conditions. Early experiments infecting npr4-1 plants with the fungi Erysiphe cichoracearum (powdery mildew) and bacterium Pseudomonas syringae pv. Tomato DC3000 (Pst DC3000) indicated that these plants were compromised in disease resistance (Liu et al., 2005). However, an independent study from Zhang and colleagues (2006), partially disagreed, rather observing that the npr4-3 and npr4-2 plants were not more susceptible to Pst DC3000 or Pseudomonas syringae pv. maculicola ES4326 (Psm ES4326). When combined with the npr3-1 mutant (npr3-1npr4-3) plants were found to be more resistant (Zhang et al., 2006). Corroborating the results of Zhang and colleagues (2006), single npr3 or npr4 mutants showed little difference in SAR response when compared to Col-0. Furthermore, the double mutant
(npr3npr4) was highly resistant in basal and induced SAR states (Fu et al., 2012). At the basal level, NPR3 deficient backgrounds have compromised fitness when measured by primary root length, average growth rates, and seed production. Most recently an npr3-3 mutant was generated and found to not differ from Col-0 plants in terms of quantity of bacterial growth when leaves were infiltrated with Pst DC3000, consistent with previous data. Conversely, transgenic plants overexpressing NPR3 were more susceptible to inoculation (Shi et al., 2013). Interestingly, the quantity of NPR3 transcripts was approximately 3-fold lower in flower petals when taken from the npr3-3 background in comparison to the npr3-2 mutant (Shi et al., 2013). Although, both backgrounds were created from homozygous T-DNA insertions in the third exon, the npr3-2 plant may nonetheless be a “weak allele” in flowers, at least (Shi et al., 2013).

2.3.2 NPR1/NPR3/NPR4, SA and the regulation of SAR: Some shortcomings

Contemporary analysis suggests that NPR4 is a CUL3 E3-ligase substrate-adapter in naïve cells, which can interact with NPR1, allowing for the continuous ubiquitylation and turnover of NPR1 by the proteasome. During SAR, the cellular accumulation of SA allows NPR4 to bind the hormone, disrupting the NPR4-NPR1 interaction and abolishing the adaptor-substrate complex. Conversely, NPR3 responds to the abundance of SA by presumably binding to the hormone allowing NPR3 to interact with NPR1, resuming ubiquitylation of NPR1, targeting it for degradation (Fu et al., 2012). Hence, NPR3 and NPR4 would function as both substrate adaptors and SA- receptors that mediate the degradation of NPR1 in the SAR induced and naïve cells, respectively.

Despite the attractiveness of this model, it has yet to be demonstrated how NPR3 or NPR4 actually interact and bind SA. Furthermore, no structural changes in these
proteins were directly observed upon binding SA. Such conformational changes are the usual hallmark of receptor-ligand interactions. In what appears to be a controversial finding, the study by Fu and colleagues (2012) suggested that NPR1 was unable to bind SA. Interestingly, using the same non-equilibrium method (see Table 1), Wu and colleagues (2012) came to the same conclusion, as they also found that NPR1 could not bind SA under these conditions. However, NPR1 clearly binds SA under equilibrium conditions when appropriate methodologies are used and chelating agents are omitted from experimental buffers (Wu et al., 2012 and Table 1). Furthermore, Manohar et al (2015), demonstrated, using three alternative methods, that NPR1 binds SA, bringing to five the total number of methods tested to demonstrate that NPR1 is an SA-receptor (Table 1). While these data clearly confirm that NPR1 binds SA, they also clearly show the need to confirm that NPR3 and NPR4 can indeed bind SA, especially given the fact that they do not undergo conformational changes upon binding SA. Therefore, considering that NPR1 is also an SA-receptor that binds the hormone with a relatively high affinity in the presence of copper, it is also unclear in vivo whether the interaction between NPR1-NPR3/NPR4 is a result of SA bound to NPR1 or to NPR3/NPR4. Since yeast-two-hybrid assays were used to study the SA-dependent interactions between NPR1 and NPR3/NPR4, it is possible that cellular copper was present at quantities sufficient to allow NPR1 to bind SA. Transient BiFC assays in naïve onion epidermal cells have also indicated an interaction between NPR1 and NPR3. However, it is unclear whether basal levels of SA were present at sufficient concentrations in the naïve onion epidermal cells to allow SA perception by NPR1 or NPR3, making it unclear whether or not the interaction requires NPR1 bound SA, NPR3 bound SA or whether the interaction requires
the presence of SA at all *in vivo* (Shi et al., 2013). However, because NPR1 has a higher affinity for SA than NPR3, as observed by the respective dissociation constants, it would follow that NPR1 would outcompete NPR3 for the interaction with SA (Fu et al., 2012; Wu et al., 2012). Given that NPR1 is the only NPR (among NPR1/NPR3/NPR4) shown to display a conformational change upon binding to its ligand, NPR1 may in fact be the decisional entity responsible for dictating whether interaction with NPR3/NPR4 occurs, regardless of the SA-status of the system.

Although NPR3 and NPR4 appear to degrade NPR1 in an SA-dependent and independent model respectively, the biochemical and phenotypic data observed from the *npr3*, *npr4*, and *npr3npr4* mutant plants are not always in agreement with this hypothesis. For example, in the *in vivo* NPR1 degradation experiment (Fig 1A in Fu et al., 2012), in the *npr4* mutant, in which the NPR3-mediated NPR1-degradation is not affected, NPR1 accumulates to the highest levels after 8h SA application. This indicates that NPR4 and not NPR3 is responsible for degrading NPR1 under SA conditions, which is not consistent with the model. Furthermore, although NPR1 accumulates to some extent in *npr3npr4* mutant before SA application, NPR1 accumulates to even greater extent in the *npr3npr4* double mutant in response to SA treatment, which indicates that the *npr3npr4* mutant is not completely insensitive to SA, suggesting that there is(are) other SA receptor(s) which mediate or trigger the accumulation of NPR1. Another indication, illustrating the presence of central receptor(s) of SA other than NPR3/NPR4, is the data showing that *Psm* ES4326 growth is significantly decreased in the *npr3npr4* double mutant plant even without SAR induction (Fig 4a in Fu et al., 2012). This does not suggest that SAR is defective as proposed by the authors, but rather that SAR is already
established in the \textit{npr3npr4} double mutant. Further inconsistencies with the model are revealed by the SAR sets of experiments. Although SA accumulation was not quantified in these experiments, treatment with the \textit{Psm avrRpt2} strain would presumably induce SAR and thus promote SA accumulation. Therefore the model would predict that, if \textit{NPR4} is a CUL3 substrate adaptor only in the absence of SA, the \textit{npr3npr4} mutant should not be more resistant that the single \textit{npr3} mutant.

On the \textit{PR1} front, the relative expression of the gene in naïve cells shows a slightly higher than wild-type induction in the \textit{npr3} plants and about the same induction as wild-type in \textit{npr4} plants. By contrast \textit{PR1} induction was several folds greater in the \textit{npr3npr4} plants when compared with wild-type or the single \textit{npr3} or \textit{npr4} mutants. The current NPR3/NPR4-NPR1 degradation model would predict rather that the \textit{npr4} plants should display similar \textit{PR1} induction as the \textit{npr3npr4} plants and that the \textit{npr3} plants should be no different from the wild-type. This is expected because of the lack of NPR3-targeted degradation of NPR1 in naïve cells. As proposed by Zhang and colleagues (2006), NPR3 and NPR4 appear to have redundant functions with respect to immunity, as opposed to the model proposed by Fu and colleagues (2012), where they have distinct non-overlapping functions.

\textbf{2.3.3 Final thoughts on the role of NPR family proteins in plant immunity}

The NPR3/NPR4-mediated NPR1 degradation is reminiscent of the emerging trend of ubiquitylation in plant hormone signaling (Santner and Estelle, 2009). Auxins act by stimulating the degradation of Aux/IAA transcriptional repressor through the ubiquitin-ligase complex \textit{SCF}^{\text{TIR1}} (Gray et al., 2001). Jasmonates activate downstream gene transcription by promoting degradation of the \textit{JAZ} family of repressors through
SCF\textsuperscript{COL1} E3 ubiquitin-ligase (Chini et al., 2007). The gibberellin receptor GID1 mediates ubiquitylation and degradation of DELLA repressor, thus activating gibberellin-responsive gene transcription (Griffiths et al., 2006). It seems that in many signaling pathways, plants use ubiquitin and the proteasome pathway to regulate the abundance of negative regulators of the corresponding system. However, in contrast to the aforementioned pathways, in the case of SA signaling, the proteasome targets the positive regulator NPR1. Although the biological importance and molecular mechanism of SA-regulated NPR1-degradation needs further investigation, ubiquitylation also plays a role in mediating SA signaling (Fu et al., 2012).

Cys521 and Cys529 responsible for the binding of SA to the \textit{Arabidopsis} NPR1 are not universally conserved in NPR1 orthologues, such as those found in crops. However, metal interaction with proteins is not limited to Cys, since any amino acid harboring electronegative elements in its side chain can potentially participate in metal interaction (Wu et al., 2012 and Figure S2 therein). Objectively, this leaves us with three possible scenarios: 1) NPR1 from crops could bind SA through metal-coordination, as does the \textit{Arabidopsis} NPR1, using amino-acids other than Cys; 2) NPR1 from crops could bind SA without coordination through a metal; 3) NPR1 from crops are not receptors for SA. Further research on crop NPR1 should prove invaluable in assessing whether, in the case of NPR1, \textit{Arabidopsis} can serve as a model system or whether it is the exception to the rule.

Since NPR1 is a transcriptional coactivator (Rochon et al., 2006), the discovery that two NPR1 family members are Cul3 substrate-adaptors (Fu et al., 2012) came as a surprise. Given that NPR3 and NPR4, just like NPR1, interact with the TGA family of
transcription factors (Liu et al., 2005; Zhang et al., 2006), a role for these proteins in transcription regulation would have been anticipated. Nevertheless, as proposed (Zhang et al., 2006), a regulatory function for NPR3 and NPR4 involving transcriptional control may still be revealed in the future.

2.4 The roles of other TGA transcription factors in plant immune system

The TGA family transcription factors consist of 10 members in Arabidopsis (Figure 1, Gatz, 2013). Based on the amino acid similarities, TGAs can be divided into five clades. Clade I, II and III TGAs are known to play roles in plant immunity, while clade IV and V have been shown to regulate plant flower development (Gatz, 2013).

On the molecular level, the best-studied TGA members are TGA2, TGA5 and TGA6 (TGA2/5/6). The TGAs of this clade act downstream of SA signaling (see 2.2.2.2 for details). Upon SA accumulation in the cells, TGA2/5/6 interact with the SA receptor and transcription coactivator, NPR1 (Wu et al., 2012). TGA2/5/6 and NPR1 form an enhanceosome on the PR1 promoter and coordinately activate the transcription of PR1 (Rochon et al., 2006).

The clade I TGA transcription factors consist of TGA1 and TGA4. These two TGAs are known to play redundant roles in the plant immune system (Shearer et al., 2012). Notably, TGA1 is a historical transcription factor in plants as TGA1 from tobacco was the first identified plant transcription factor (Katagiri et al., 1989). Since the identification of TGA1/4, they have been shown to be positive regulators of basal immunity and negative regulators of the SAR maker gene, PRI (Lindermayr et al., 2010; Shearer et al., 2012). However, the molecular mechanism of TGA1/4 is unknown.

TGA1/4 are particularly intriguing to study for two reasons. First, despite the fact
that TGA1/4 repress the PRI transcription, TGA1/4 are known to interact with NPR1 in vivo (Després et al., 2003). This means that the same family of transcription factors (TGA2/5/6 and TGA1/4) interacts with the same transcriptional coactivator (NPR1), and yet, they have opposite output on gene regulation. Second, TGA1/4 are the only TGAs that are known to control both basal immunity and SAR. For the reasons above, chapter 3 is dedicated to the study of TGA1/4.

2.5 Methods developed or adapted to study plant immune system in this thesis

The methods developed or adapted to study plant immune system in this thesis include two large-scale analyses of RNA-sequencing (RNA-seq) data and one biophysical analysis of proteins. A comparison of conventional methods and methods developed or adapted in this thesis is shown in Table 2. The development or adaptations of these methods in studying the plant immune system are not intended to replace the current technologies. They are simply meant to offer alternative ways to avoid exhaustive use of the current methods. In addition, the use of novel research tools can provide different insights to the increasingly complex understanding of the plant immune system.

2.5.1 Maximizing the use of RNA-seq data: large-scale analyses of plant immune system

With the fast development and the lower-cost of high-throughput sequencing, RNA-seq has become a standard procedure in various research fields. In plant immunity research, RNA-seq or microarray experiments are primarily used to determine deferentially regulated genes (DEGs) of a particular protein target or DEGs in response to a certain stimulus (Zipfel et al., 2004; Shearer et al., 2012). The determination of DEGs controlled by a protein is achieved through comparative analyses of the transcriptome
profiles of WT plants and the protein target knockout or knockdown mutant, such as T-DNA insertion line or RNA interference line. DEGs in response to a stimulus can be determined by comparing the transcriptomes of plants with the same genotype before and after the exposure to a stimulus. In studies of the plant immune system, RNA-seq has provided a wealth of information on the genes controlled by an individual protein or stimulus. The number of DEGs is often used as an evidence of the importance of a protein or stimulus. However, little information is extracted from the actual content and the meaning of the DEGs. In this thesis, I adapted the ClueGO analysis platform, which can create a functionally organized gene ontology (GO) term network, to visualize plant transcription factor-controlled processes in a network format (Chapter 3). Moreover, a transcription factor-coregulator identification strategy based on transcriptome analyses is also developed in this thesis (Chapter 3). Both of these two large-scale analyses can be used to expand the standard Arabidopsis RNA-seq data analyses pipeline and extract valuable information from RNA-seq data using unconventional ways.

2.5.1.1 ClueGO analysis

After determining DEGs of a protein or in response to a stimulus, GO analysis is the go-to procedure for studying the function of the DEGs. GO is a bioinformatics initiative that aimed to produce a unified vocabulary of gene and gene product attributes that can be applied to all eukaryotes (Ashburner et al., 2000). GO annotates genes to three independent ontology categories: biological process, molecular function and cellular components. Within each category, genes can be annotated to GO terms in a hierarchically structured way. Biological process is defined as a biological objective, to which the gene or gene product contributes, for examples, the term “response to salicylic
acid”. Molecular function refers to the biochemical activity of a gene product such as “kinase activity”. Cellular component is designed to represent where in the cell a gene product is active such as “nuclear membrane”. Perform GO analysis with a list of DEGs under biological process category can tell us what are the biological processes input DEGs are involved in.

GO analysis is a great method to systemically capture biological information from a large set of data. However, in many cases, the representation of a GO analysis result can be a long list of terms or a complex hierarchical tree. Take GO analysis (biological process) of DEGs down regulated by plant immune transcription factors TGA1/4 as an example. 1005 DEGs were used as input for the GO term analysis performed by the agriGO. AgriGO is a web-based tool and database for GO analysis, specialized in agriculture and model plant species (Du et al., 2010). Even when GO terms are only selected with p-value<0.0005, the result of GO analysis on DEGs negatively regulated by TGA1/4 is represented as a list of 249 GO terms or a hierarchical tree consist of 249 items. Such representations make the interpretation of the data very difficult.

ClueGO is a Cytoscape plugin, which can generate a functionally organized GO term network (Bindea et al., 2009). In the case of DEGs negatively control by TGA1/4, instead of an exhaustive list of terms, the GO analysis result is represented as functionally grouped terms in the form of a network (Figure 1a). 10 enriched groups are generated for DEGs negatively controlled by TGA1/4. Such visualized representation allows us to easily conclude that the two major biological processes controlled by TGA1/4 are “cell death” and “defense response”.

How does ClueGO transform a list of 249 terms to a visualized network consisted
of 10 enriched groups? First, to eliminate term redundancy, GO terms in the same parent–child relation sharing similar associated genes are first identified. Only the most representative parent/child term is preserved. Second, to determine the relationships between terms, the kappa statistic is used to calculate the association strength between terms based on their overlapping genes (Huang et al., 2007). Finally, to generate a visualized network, GO terms are represented as nodes, which are grouped and linked based on kappa score level. The node size represents the term enrichment significance. The most significant term in a group is assigned as group name. As such, a visualized network representation of GO analysis result is generated. ClueGO allows researchers to interpret data in a more comprehensive and visual way with minimal redundant information.

ClueGO analysis can also be used to compare functional annotations of two lists of genes. Take the comparison of DEGs controlled by TGA1/4 and NPR1 as an example. As it is shown in Figure 1b, the GO terms only controlled by TGA1/4 or NPR1 are represented as blue and yellow respectively and the terms co-controlled by TGA1/4 and NPR1 are shown in grey. We can determine that TGA1/4 are tentacular transcriptional repressors involved in the control of a large number of biological processes, with a much greater functional breadth than NPR1.

2.5.1.2 A transcription factor-coregulator identification strategy based on transcriptome analyses

The genome is the fundamental component of all living organisms. But what governs the proper decoding of our genetic information? One such player is the transcription factor. A transcription factor is a protein that controls the rate of DNA being
transcribed into messenger RNA by binding to a specific DNA sequence (Lodish et al., 2000). Intriguingly, in many cases, transcription factors do not work alone. They require coregulators to control the rate of transcription. Transcription coregulators are proteins that interact with transcription factors to either activate or repress the transcription of specific genes (Lodish et al., 2000). Identifying the coregulator of a transcription factor has profound clinical importance for both animals and plants. This is because proper cellular functions and activation of immune systems often require proper actions of transcription factors together with coregulators. Conversely, many diseases happen because of the malfunction of transcription factors and their coregulators (Lee and Young, 2013). This makes coregulators excellent drug targets for both animals and plants. Therefore, identifying coregulators is a critical step for maintaining healthy lives using pharmaceutical approaches. Currently, methods for identification of coregulators mainly include protein-protein interaction based assays (Despres et al., 2000). However, this type of assay is very labor-intensive and time consuming.

In this thesis (Chapter 3), a novel, yet efficient, transcription factor-coregulator identification method using a combination of genetics and bioinformatics tools is developed. Using this method, a protein GRX480 was identified as the potential coregulator of crucial plant immunity regulating transcription factors, TGA1/4. Most excitingly and importantly, we have demonstrated that GRX480 indeed can act as the coregulator of TGA1/4 in vitro and in vivo.

2.5.2 The use of biophysical method to discover plant immunity-activating agents

The goal of studying the molecular mechanisms of the plant immune system in depth is to translate this knowledge into crop protection strategies. Two major ways can
achieve this goal: developing plant immunity-activating agents and facilitating breeding technologies to select resistant cultivars (Kuai and Despres, 2016; Kuai et al., 2017). Currently, phenotype-base screens are the main tool used in the development of plant immunity-activating agents (Knoth et al., 2009; Noutoshi et al., 2012). However, such technology cannot fully take advantage of the advancements we have made in understanding molecular mechanisms. For example, NPR1 was identified as a master regulator of plant immunity since 1997. Despite the great molecular knowledge we have gained on this protein, no crop protection strategy has been developed based on NPR1. Chapter 4 showcases a target based biophysical method, differential scanning fluorimetry, which has promising potential to be used for developing plant immunity-activating agents.
Table 1. Comparison of SA-binding properties between NPR1, NPR3 and NPR4.

<table>
<thead>
<tr>
<th>Method used to study SA-binding</th>
<th>NPR1</th>
<th>NPR3</th>
<th>NPR4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Equilibrium dialysis (Non-equilibrium methods not working suggesting fast on/off rates)</td>
<td>Conventional non-equilibrium ligand binding assay (Slow off rates)</td>
<td>Conventional non-equilibrium ligand binding assay (Slow off rates)</td>
</tr>
<tr>
<td>Affinity</td>
<td>$K_d = 140 \pm 10 \text{ nM}$ (High affinity)</td>
<td>$K_d = 981 \pm 409 \text{ nM}$ (Low affinity)</td>
<td>$K_d = 46.2 \pm 2.35 \text{ nM}$ (High affinity)</td>
</tr>
<tr>
<td>Secondary binding method</td>
<td>Scintillation proximity assay (Wu et al., 2012). Surface Plasmon Resonance, photoaffinity labeling, and size-exclusion chromatography (Manohar et al., 2015)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Ligand Interface</td>
<td>Cys$^{521/529}$</td>
<td>Not known</td>
<td>Not known</td>
</tr>
<tr>
<td>Stoichiometry</td>
<td>-SA: Oligomer</td>
<td>Not known</td>
<td>-SA: Tetramer</td>
</tr>
<tr>
<td></td>
<td>+SA: Dimer</td>
<td></td>
<td>+SA: Tetramer</td>
</tr>
<tr>
<td>Conformation change and molecular properties after SA-binding</td>
<td>-SA: N-terminal BTB domain interacts with C-terminal transactivation domain to inhibit the transcription activity of NPR1. +SA: Disruption of the interaction between BTB and C-terminus converting NPR1 into a transcription coactivator.</td>
<td>No conformation change known. -SA: Does not interact with NPR1. +SA: Interacts with NPR1.</td>
<td>No conformation change known. -SA: Interacts with NPR1. +SA: Does not interact with NPR1.</td>
</tr>
<tr>
<td>Metal requirement for SA-binding</td>
<td>Requires copper</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Crystal structure</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
</tbody>
</table>
Table 2. Comparison of conventional methods and the methods developed or adapted to study plant immune system in this thesis.

<table>
<thead>
<tr>
<th>Methods to analyze the functions of a list of genes</th>
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<tbody>
<tr>
<td>Method</td>
<td>Purpose</td>
</tr>
<tr>
<td>GO analysis</td>
<td>Provide all processes controlled by a list of genes.</td>
</tr>
<tr>
<td>ClueGO analysis</td>
<td>Provide all processes controlled by a list of genes. Group GO terms according to the functions. Visualize functionally grouped GO in the form of a network.</td>
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<tr>
<th>Methods to identify transcription factor-coregulator</th>
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<tr>
<td>Method</td>
<td>Assay nature</td>
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<tr>
<td>Yeast two-hybrid screening</td>
<td>Biochemical screening</td>
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<tr>
<td>Transcriptome analyses based method</td>
<td>RNA-seq data based identification</td>
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<th>Methods to discover immune activating agents</th>
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<tbody>
<tr>
<td>Method</td>
<td>Assay nature</td>
</tr>
<tr>
<td>Phenotypic screening</td>
<td>Biochemical assay</td>
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<tr>
<td>Thermal shift assay</td>
<td>Biophysical assay</td>
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</table>

Methods highlighted in blue are conventional methods used to study the plant immune system. Methods highlighted in red are the methods developed or adapted, in this thesis, to study plant immune system.
Figure 1. Clustering of *Arabidopsis* TGA Transcription Factors

Figure 2. ClueGO example analyses of TGA1/4 down regulated genes and NPR1 up regulated genes

a. Gene ontology map using *tga1/tga4>*WT DEG. The significance of each term is calculated using two sided hypergeometric test and p-value is corrected using Bonferroni method. GO term enrichment were selected when p-value < 0.05. GO terms are represented as nodes which are linked based on kappa score level (≥0.3). The node size represents the term enrichment significance. Only the most significant term in the group was labeled. The group color are randomly assigned by ClueGO. 

b. Gene ontology map using *tga1/tga4>*WT DEG and *npr1>*WT DEG. GO terms specific to TGA1/4 are shown in blue, NPR1-specific ones in yellow and GO terms common to both TGA1/4 and NPR1 are in grey.
2.6 REFERENCE


Chanda, B., Xia, Y., Mandal, M. K., Yu, K., Sekine, K. T., Gao, Q. M., ... & Kachroo, A.
Glycerol-3-phosphate is a critical mobile inducer of systemic immunity in plants. Nature genetics, 43(5), 421.


homology to the nuclear factor CREB. *Nature, 340*(6236), 727.


283, 996–998.


CHAPTER 3 - A clade of plant transcription factors antagonizes the immune control of two major phytohormones through distinct molecular mechanisms.

Contributions

I initiated, designed and performed the experiments in Figure 1, 2, 4, 5, 7, Figure 6 a, b, c, d, Table 1, Supplemental Figure 1, 4, 5, 7 and Supplemental Table 1, 2 under the guidance of Dr. Després. I wrote the first draft of the manuscript.

Xiahezi Kuai1, Amanda Rochon1,3, Yue Wu1,4, Charles Barraco1, Nina Slavickova1, Marina Pombo2, Hernan Rosli2 & Charles Després1*

1Department of Biological Sciences, Brock University, 1812 Sir Isaac Brock Way, St. Catharines, Ontario, Canada, L2S 3A1.
2Instituto de Fisiología Vegetal (INFIVE, CONICET-UNLP), La Plata 1900, Argentina.
3Present address: School of Health and Wellness (Biotechnology - Advanced), Fleming College, 599 Brealey Drive, Peterborough, Ontario, Canada, K9J 7B1.
4Present address: Department of Molecular Biology, Massachusetts General Hospital, and Department of Genetics, Harvard Medical School, Boston, Massachusetts, 02114, USA.

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Author Contributions X.K. and C.D. initiated and designed the research. X.K., C.D., analysed data and wrote the manuscript. X.K. conducted the RT-qPCR and CHIP experiments in Figure 2 and Figure 5 and generated all the large-scale data-sets under the guidance of H.R. and C.D. A.R. performed pull-down, transcription and two-hybrid assays, Y.W. generated and analyzed RNAi lines and performed RT-qPCR in Figure 8, C.B. performed the ROS measurements and N.B. performed ChIP experiments in Figure 8.
3.1 INTRODUCTION

Multicellular land plants are sessile organisms, and as such are exposed to the environment, which includes threats from pathogens. As a mechanism to cope with microbes, each plant cell has the capacity to perceive and deploy a local immune response, which in some cases can lead to the propagation of signals throughout the plant for the deployment of a systemic immune response termed systemic acquired resistance (SAR) (Jones and Dangl, 2006). The type of pathogen, whether virulent or avirulent, perceived locally will dictate the nature of the immune response. Virulent pathogens can trigger a local-only basal immunity, which is brought about by the detection of pathogen-associated molecular patterns or PAMPs (Chisholm et al., 2006). An example of PAMP is a stretch of 22 amino acid found in the protein flagellin, which is a structural component of the flagellum found in some pseudomonads (Zipfel, 2014). This peptide termed flg22 can on its own turn on the basal immunity (Chinchilla et al., 2006). A hallmark of basal immunity is the production of reactive oxygen species (ROS), which have direct antimicrobial activities, but can also act as signaling molecules within plant cells (Lozano-Durán et al., 2013; Baker and Orlandi, 1995). Avirulent pathogens can trigger both local immunity followed by the distal SAR (Jones and Dangl, 2006). The local immunity is triggered by the perception of effector molecules, which are often delivered intracellularly from the pathogen to the plant. This effector-triggered immunity leads to the production of signals required for the establishment of SAR. Hallmarks of SAR are the requirement for salicylic acid (SA) and the systemic induction of pathogenesis related (PR) genes (Ward et al., 1991; Vlot et al. 2009). A treatment with SA is sufficient for the deployment of SAR, which is best studied molecularly through
the transcriptional control of the SAR-marker gene *PRI* (Zhang et al., 2003; Rochon et al., 2006; Boyle et al., 2009). Once deployed, SAR is effective against a broad range of pathogens (Vlot et al. 2009). Basal immunity and SAR coordinately protect plants against pathogen attack through different signaling pathways. However, the one underlying theme common to both types of immunity is the necessity of transcriptional control (Eulgem, 2005).

Transcriptional regulation of gene expression relies on the recognition of promoter elements by transcription factors (Mitchell and Tjian, 1989). These regulatory proteins are generally part of families containing structurally related members displaying similar or identical DNA binding site preferences. Such families may contain transcriptional activators and repressors as well as dual-function transcription factors capable of gene activation in one context and repression in another. There are, in any given organism, fewer genes encoding transcription factors than there are genes to regulate. Furthermore, each gene may be regulated individually by different stimuli, with differing timing and in specific tissues or even cell-types. This complexity can be handled to some degree through the genetic and molecular interactions of transcription factors found within a family or between factors of different families. It can be further handled by the recruitment of coregulators by transcription factors. In plants, two major families of transcription factors ensure the transcriptional regulation of the plant immune system; the WRKY and TGA families (Pandey and Somssich, 2009; Gatz, 2013). Members of both WRKY and TGA families control various aspects of immunity. However, little is known about the intertwined relationship, if any, between WRKYS and TGAs. In plants, both forward and reverse genetics has provided a wealth of information on the genes
controlled by individual transcription factors. However, at the gene level, detailed mechanistic regulation of gene expression is not as broadly understood in plants as it is in animals. For instance, no regulation model of a plant gene is as well developed as the textbook example of the interferon-beta enhanceosome. Furthermore, on a pathway or a system scale, it is unclear how the different families of transcription factors and coregulators integrate together to control specific systems.

The Arabidopsis TGA family of transcription factors contains 10 members (Gatz, 2013). Transcription factors TGA1 and TGA4 are the only members of clade I and both play redundant roles in controlling basal immunity (Shearer et al., 2012). This makes them particularly appropriate to study the interrelationship between the TGA and WRKY families, with several WRKYs being also involved in the regulation of basal immunity (Chisholm et al., 2006). The clade II TGA contains three members (TGA2, TGA5, TGA6), which play redundant role in the deployment of SAR (Zhang et al., 2003). As the concentration of SA builds-up after a local interaction with an avirulent pathogen, SA is recruited by the coactivator and SA-receptor NPR1 (Wu et al., 2012). This leads to the formation of an enhanceosome composed of Clade II TGA and NPR1. Recruitment of the enhanceosome to the promoter of the SAR marker gene PR1 leads to its activation (Rochon et al., 2006). Intriguingly, clade I TGA has been found to negatively regulate PR1, in spite of their capacity to recruit NPR1 (Després et al., 2003; Lindermayr et al., 2010). This makes TGA1 and TGA4 particularly interesting for studying the mechanism that allows these seemingly similar clades of TGA (clade I and II) to perform diametrically opposite function with respect to the regulation of the SAR marker gene PR1.
TGA1 was the first transcription factor to be identified in plants (Katagiri et al., 1989). Since its discovery, multiple genetic and phenotypic studies have revealed the critical roles of TGA1/4 in nitrate response and immune responses (Shearer et al., 2012; Alvarez et al., 2014). However, despite the fact that clade I TGA holds this historical position, questions abound regarding their molecular mode-of-action in terms of the various transcriptional mechanisms operating at individual genes or how they control and interconnect with pathways and systems. Specifically, how do the TGA1/4 fit into the well-studied WRKY-controlled basal immunity? And secondly, how do the TGA1/4 integrate into the NPR1-centered SAR network and what is the mechanism that diametrically opposes clade I and clade II TGA?

3.2 RESULTS

3.2.1 TGA1/4 positively regulate basal immunity by repressing negative regulators of the process

Given that TGA1/4 positively controls basal immunity, while negatively regulating components of SAR, highlight the complexity of the immune response circuitry and poses a formidable challenge to the development of crop protection strategies based on modulating plant immunity. Driven by a global need for such agricultural strategies, we set out to address the seemly contradictory roles, played by these historical transcription factors, in modulating immunity. We first performed RNA sequencing experiments on WT and tga1tga4 double mutant plants after a short 4-h treatment with SA. The intent was to capture both basal immunity- and early SAR-response genes and reveal some of the molecular switches controlled by TGA1/4 and
involved in one of the two processes.

Gene ontology (GO) analysis, using ClueGO, for processes associated with positive and negative regulation of gene expression by TGA1/4 is shown in Figure 1. It is very important to realize that these GO processes can contain both entities that positively or negatively regulate such processes. We found that processes related to immunity are associated with negative regulation of gene expression by TGA1/4. Given that basal immunity is positively regulated by TGA1/4, one must conclude that TGA1/4 controls negative regulators of basal immunity. Negatively regulating a negative process is likely a more complex scenario that could be best tackled through a cascade of transcription factors, where TGA1/4 would control a small number of transcription factors themselves negative regulators of immunity.

Because of the well-established role of WRKY transcription factors in both the positive and negative regulation of basal immunity, we first looked whether TGA1/4 control any WRKYs. Our transcriptome analysis revealed that TGA1/4 control 9 WRKYs (q<0.01) (Table1). All nine WRKYs were found to be repressed by TGA1/4. We next scanned the literature to identify whether any of the 9 WRKYs play clearly demonstrated roles in immunity, such as through the use of a knock-out line. Indeed, three of the 9 WRKYs, WRKY 15, WRKY 40 and WRKY48 (hereon referred to as WRKY15, 40, 48) are known to be negative regulators of basal immune responses (Table1). The repressing effect of TGA1/4 on these three WRKYs was confirmed by RT-qPCR (Figure 2 a, b, c). We next performed CHIP-qPCR to determine whether the regulation of these WRKYs by TGA1/4 is direct or indirect. The results showed that TGA1/4 can directly bind to the promoters of all three WRKYs (Figure 2 d, e, f). Therefore, our results support a model in
which TGA1/4 could positively regulate basal immunity by directly binding and repressing a subset of WRKYs that negatively regulate this process.

3.2.2 The segment of basal immunity regulated by brassinosteroids is co-controlled by TGA1/4

The identification of a subset of WRKYs as molecular switches controlled by TGA1/4 revealed an unexpected but significant connection between TGA1/4 and brassinosteroids. Indeed, the three WRKYs identified as direct targets of TGA1/4 are also known direct targets of the BZR1 transcription factor (Sun et al., 2010). BZR1 is the master regulator of the well-established brassinosteroid-regulated growth and basal immunity trade-off. Culminating in the activation of BZR1 after activation of the trade-off signaling pathway by brassinosteroids, BZR1 suppresses basal immunity by activating the expression of several WRKYs, including WRKY15, and WRKY48, that are negative regulators of the process (Lozano-Durán et al., 2013). In addition to controlling WRKY gene expression, BZR1 also physically associates with WRKY40 to repress basal immunity (Lozano-Durán et al., 2013). The connection between BZR1 and TGA1/4, through their common binding to the same WRKY promoters, prompted us to investigate the relationship between TGA1/4 and brassinosteroid-regulated genes. A comparison between all known brassinosteroid-regulated genes, found in a previously published database compiled from 10 microarray studies, and TGA1/4-regulated genes, identified in this study, revealed that 273 genes are co-regulated by brassinosteroids and TGA1/4 (Figure 3a, Sun et al., 2010). A ClueGO analysis showed that the process associated with the term “immune responses” was found within the subset containing the 273 genes co-regulated by TGA1/4 and brassinosteroids (Figure 3b), but that no such
term was found within the brassinosteroid-only subset (Figure 3c). In contrast, the term
“immune responses” was also found in the TGA1/4-only subset, indicating that the
regulation of immune processes by TGA1/4 is both brassinosteroid-dependent and
independent (Supplemental Figure 1). This is in-line with their involvement in basal
immunity, but also in SAR. These results strongly indicate that the segment of basal
immunity negatively regulated by brassinosteroids is also under the control of TGA1/4,
which acts as a dampener to allow or disallow suppression of basal immunity by
brassinosteroids.

3.2.3 TGA1/4 antagonize the brassinosteroid-dependent suppression of basal
immunity

Thus far, a model is developing in which the brassinosteroids/BZR1-controlled
growth-immunity trade-off is co-controlled by TGA1/4. To confirm the bioinformatics
results and to further investigate the role of TGA1/4 in brassinosteroids-suppressed basal
immunity, we tested the production of reactive oxygen species (ROS) after flg22
treatment in naïve conditions and after pharmacological activation or blockade of the
brassinosteroids signaling pathway leading to BZR1 activation (Figure 4a). This approach
is well established and accepted as a proxy for basal immunity. The base experiment
demonstrating the effect of the pathogen-associated molecular pattern (PAMP) molecule
flg22 on ROS production appears in Supplemental Figure 2. To pharmacologically
modulate the brassinosteroids pathway, we used the well-established pathway inhibitor,
brassinazole, and pathway activator, bikinin (Figure 4a) (Asami et al., 2000; De Rybel et
al., 2009). As a control, we first tested and confirmed the effect of brassinosteroids on
basal immune responses using wild-type plants. Activation of the brassinosteroids
signaling pathway by treatment with bikinin caused a significant reduction of ROS production, confirming that brassinosteroids repress basal immunity (Figure 4b). Conversely, inhibition of the brassinosteroids signaling pathway by treatment with brassinazole led to a significant increase in ROS production, confirming again that brassinosteroids repress basal immunity (Figure 4c). Next, we investigated the role of TGA1/4 in brassinosteroids-mediated suppression of basal immunity using the tga1tga4 double mutant. Following the schematics of Figure 4a, activating the brassinosteroids signaling pathway by treatment with bikinin will lead to an increase activity of BZR1, which will lead to an increase activity of the WRKYs and a decrease in ROS production. Removing TGA1/4 from the system allowed for an even further and statistically significant reduction in ROS production (Figure 4d, e). This result demonstrates that TGA1/4 are negative regulators of brassinosteroids-mediated suppression of basal immunity. In a more intuitive experiment, the inhibition of the brassinosteroids pathway by brassinazole, which leads to increase ROS production in the wild-type, completely abolished the increased ROS production in the tga1/4 double mutant (Figure 4f, g). This observation clearly demonstrates that TGA1/4 are required to dampen the brassinosteroids-mediated suppression of ROS production. Taken together, the results of activating and inhibiting the brassinosteroids pathway and hence modulating ROS production, suggest that TGA1/4 are required to dampen the brassinosteroids-mediated suppression of basal immunity.

3.2.4 TGA1/4 physically integrate into the growth-immunity tradeoff at WRKY promoters

Since brassinosteroids-mediated suppression of immunity is dependent on
WRKYs, we investigated whether TGA1/4 can regulate WRKYs during the modulation of the brassinosteroids-pathway. RT-qPCR results show that TGA1/4 can still repress the transcription of WRKY15, 40, 48 even after brassinazole treatment. (Figure 5a, b). This result is consistent with a model in which TGA1/4-dependent increase in ROS production, observed in wild-type plants treated with brassinazole (Figure 4), operates through the repression, direct or indirect, of the WRKYs. To test whether the control of TGA1/4 on the WRKYs is direct, we performed chromatin immunoprecipitation (ChIP) experiments and compared the enrichment of TGA1/4 in the wild-type versus the tga1/tga4 mutant. The ChIP results showed enrichments of TGA1/4 on the WRKY15, 40, 48 promoters specifically after brassinazole treatment (Figure 5c, d, e), indicating the direct control of TGA1/4 over the subset of WRKYs regulating the brassinosteroids-dependent suppression of basal immunity. Finally, as per the schematics of Figure 4a, we tested whether TGA1/4 could integrate at other points in the growth-immunity trade-off to control growth parameters in addition to basal immunity. A well-established experiment, to demonstrate the role of brassinosteroids on growth, is to treat wild-type plants with brassinazole and observe the restricted growth of hypocotyl (Supplemental Figure 3; WT) (Chory et al., 1991). Furthermore, we used a mutant (bzr1-1D) known to be insensitive to brassinazole and obtained the expected results, which is that hypocotyl lengths were not significantly affected by the treatment (Wang et al., 2002). Finally, we compared hypocotyl length of naïve and brassinazole-treated tga1/tga4 mutant plants and observed the normal restricted growth associated with a wild-type phenotype (Supplemental Figure 3; tga1/tga4). This observation is consistent with the bioinformatics results demonstrating that the brassinosteroid-TGA1/4 co-regulated genes
are not statistically involved in growth related processes. Collectively, our results demonstrate that TGA1/4 only integrate into the growth-immunity trade-off to regulate the immunity component by antagonizing the suppressive effects of brassinosteroids, which is achieved through the negative regulation of a subset of WRKY transcription factors (Figure 9a).

3.2.5 TGA1/4 are global transcription repressors of NPR1 processes

After investigating the interconnectivity of the TGA1/4 and the WRKY family in the control of basal resistance, we went on to address the mechanism involved in the negative regulation by TGA1/4 of the SAR marker gene *PRI*. We first looked at our transcriptome data to determine whether there were, in addition to *PRI*, other SAR-associated genes negatively regulated by TGA1/4. Out of the 1600 differentially expressed genes (DEG), 63% (1005) of them were up-regulated in the double mutant (Figure 6a, red points), indicating that TGA1/4, directly or indirectly, regulate gene expression mainly in a negative way. Furthermore, when comparing the total output in gene expression of all DEG in the double mutant versus the wild-type, we could determine, using a box plot, that TGA1/4 act predominantly as genome-wide transcriptional repressors (Figure 6b). This is indicated by the second quartile being above the threshold of 1 (red line). NPR1 activates a number of SA-dependent immune genes involved in SAR including the SAR-marker gene *PRI*. To assess whether TGA1/4 negatively regulate genes involved in SAR other than *PRI*, we comparatively analyzed our data with a transcriptome analysis previously performed on the *npr1* mutant in similar SA-treated conditions (Supplemental Figure 3) (Shearer et al., 2012). We observed a strong negative correlation between co-regulated genes up-regulated in the *tga1/tga4*
double mutant and down-regulated in the npr1 mutant (Figure 6c). Contrastingly, genes
down-regulated in the tga1/tga4 double mutant and up-regulated in the npr1 mutant did
not correlate well (Figure 6c). These data confirm that TGA1/4 negatively regulate a set
of NPR1-regulated genes, which is broad and not only limited to the PR1. Indeed, 128
genes, including PR1, are positively regulated by NPR1 and negatively regulated by
TGA1/4 (Supplemental Figure 4). Furthermore, when comparing the total output in gene
expression of the TGA1/4 and NPR1 co-regulated genes, we ascertained, using a box
plot, that TGA1/4 act predominantly as genome-wide transcriptional repressors of NPR1-
regulated genes (Figure 6d). Again, this is indicated by the second quartile being above
the threshold of 1 (red line).

3.2.6 A corepressor other than NPR1 is likely involved in the TGA1/4-repression of
NPR1-regulated genes

Given that NPR1 can interact with TGA1/4 in plant two-hybrid assays, a simple
model to explain the repression of NPR1-regulated genes by TGA1/4 would be that those
transcription factors recruit NPR1 to form a repressosome. Although NPR1 can act as a
transcriptional coactivator by forming an enhanceosome with clade II TGA (specifically
demonstrated with TGA2), certain animal coregulators have been shown to be
corepressors under one condition and be coactivators in another. We tested this model
using a previously established plant transcription activation assay. The assay is
essentially a plant two-hybrid assay in which one of the members lacks the synthetic
transcriptional activation domain. It is also noteworthy to mention that in this assay few
cells actually up-take the plasmids, but those that do produce protein levels in excess of
the native conditions. This is an advantage that allows one to interrogate an interaction,
while diminishing the effect of endogenous proteins, found in limiting concentrations, on that interaction. Surprisingly, in the plant transcription assay, we observed that in SA-treated cells, TGA1 forms an enhanceosome with NPR1 (Figure 6e). Mutations of C521 and C529 are known to abolish the transactivation potential of the transactivation domain of NPR1 without affecting NPR1’s capacity to interact with a TGA (TGA1 in Figure 6f and TGA2 in Rochon et al., 2006). The transcription assay also revealed that the transcription activation property of the TGA1-NPR1 complex was dependent on the transactivation domain of NPR1 (Figure 6e) (Rochon et al., 2006). This behavior is essentially identical to that of the TGA2-NPR1 enhanceosome, yet clade I and clade II factors are not redundant in their immune function (Rochon et al., 2006). Given that a TGA1/4-NPR1 enhanceosome has the potential to assemble (Figure 5g), the only plausible model to account for the opposite regulation of TGA1/4 and NPR1 is that TGA1/4-NPR1 enhanceosome is precluded from forming under native conditions by a yet to be identified corepressor (Figure 5h).

3.2.7 A transcriptome-based strategy identifies potential coregulators of TGA1/4

Since some transcription factors, such as NF-κB, control their own regulator (IκB) (Scott et al., 1995), we next strategized that a transcriptome analysis-based procedure would identify potential TGA1/4 coregulators capable of disrupting a TGA1-NPR1 interaction (Supplemental Figure 5). We filtered the 1600 TGA1/4 DEG searching for classic transcription factor-regulators or modifiers (categories shown in Figure 7a). We identified 143 potential candidate regulators, all of which belonged to the modifier categories and none were found associated with the corepressor groups. Of the candidate regulator found, 66% of them (95) were up-regulated in the tga1/tga4 double mutant.
(Figure 7a). This bias towards the candidate genes being negatively regulated by TGA1/4 is in-line with clade I factors having genome-wide repressing activity. As such, it is within these up-regulated candidate genes that a coregulator would be expected to be found, since these genes are more likely to be directly controlled by TGA1/4. Nevertheless, to reduce the number of potential candidates to a more manageable number, we further filtered the list of all 143 genes using keywords related to immunity. This group of keywords proved very effective in reducing the number of potential candidate. Indeed, we found only 10 genes satisfying the filtering criteria, all of which were up-regulated in the *tga1/tga4* double mutant (Figure 7a, Supplemental Table 1), indicating that TGA1/4 negatively regulate these genes. Two genes (GRXS13 and GRX480), from the protein disulfide oxidoreductase/thioredoxin category, really stood out as obvious choices to pursue because of their known interaction in yeast-two hybrid with members of the TGA family of transcription factors and because of their clear involvement in immunity (Li et al., 2009; Gutsche et al., 2015; La Camera et al., 2011). We decided to pursue GRX480 based on its lower q-value.

3.2.8 **GRX480 is a transcription corepressor of TGA1/4-dependent NPR1-regulated genes**

Since no satisfactory knock-out lines could be found for the GRX480 gene in the SALK database, we created RNA interference lines and selected one with negligible accumulation of GRX480 transcript (Supplemental Figure 6a) to perform RNA-Seq. The RNAi lines did not show any remarkable phenotypic differences compared to the wild-type (Supplemental Figure 6b). We found 300 DEG, with 61% (184) of them up-regulated in the RNAi line (Figure 7c red points). Like TGA1/4, GRX480 imparts mainly
transcriptional repression on the genome (Figure 7d). This observation is important, as it is consistent with a model in which TGA1/4 and GRX480 form a repressosome. However, for this model to hold true, a set of genes co-regulated by both GRX480 and TGA1/4 need to be detected. Strikingly, 65% (119) of the up-regulated genes, found in the GRX480 knock-down line, are also co-regulated by TGA1/4, signifying that this member of the novel glutaredoxin family is highly dedicated to functioning with TGA1/4 (Figure 7b). These data therefore support the repressosome model. Next, we addressed whether GRX480 co-regulates a subset of NPR1-regulated genes, a mandatory requirement for a repressosome aimed at down-regulating NPR1-dependent gene expression. We identified 33 co-regulated genes. Subsequently, we demonstrated that a strong negative correlation exists between 25 of the co-regulated genes up-regulated in the grx480 RNAi line and down-regulated in the npr1 mutant (Figure 7e), but that in contrast, genes down-regulated in the grx480 RNAi line and up-regulated in the npr1 mutant showed no correlation (Figure 7e). This result indicates that GRX480 is a negative regulator of a set of NPR1-regulated genes. Furthermore, when comparing the total output in gene expression of the GRX480 and NPR1 co-regulated genes, we established, using a box plot, that GRX480 act predominantly as a genome-wide transcriptional repressor of a set of NPR1-regulated genes. This conclusion is supported by the second quartile being above the threshold of 1 (red line) (Figure 7f). Of this set of 25 genes, 23 of them, or 92%, are also negatively regulated by TGA1/4, suggesting that a repressosome composed of TGA1/4 and GRX480 likely interferes with the expression of these NPR1-dependent genes (Figure 7g). We scanned the literature and uncovered the importance of these genes in regulating key aspects of immunity (Supplemental Table 2).
Next, we quantified their importance by gene ontology (GO) analysis using ClueGO (Figure 7h). Although the term “immunity” did not appear as a process regulated by these 23 genes, the term “response to molecules of bacterial origin” clearly indicate the relevance of this set of genes in the control of immunity, at least that targeting bacterial pathogens. The absence of enrichment in the term “immunity” could be due to the small number of genes being analyzed. Of particular interest, we found among these 23 genes, the classic immunity marker gene \( PR1 \) (Supplemental Table 2). Finally, the repression effect of GRX480 observed on these 23 genes was further verified by measuring transcript accumulation in 4 of these genes in another grx480 RNAi line (Supplemental Figure 7).

### 3.2.9 TGA1-GRX480 repressosome impacts NPR1-dependent co-activation

We have established that at least 23 genes can be negatively regulated by TGA1/4 and GRX480 and positively regulated by NPR1. The next step was then to determine whether the TGA and the GRX would serve as repressive entities alone or in a complex. We initially tested the capacity of TGA1 and GRX480 to interact in a classic co-immunoprecipitation assay. We observed no interaction between the two proteins (Figure 8a). This finding was not a complete surprise since TGA2 and GRX480, which interact in the yeast two-hybrid system do not interact in pull-down assays (Ndamukong et al., 2007). It has been speculated that some additional components may be required for the interaction to occur (Ndamukong et al., 2007). We hypothesized that since TGA1 and GRX480 would function at a promoter, their interaction may only occur on DNA. We have observed that some recombinant TGA factors form high-order complexes in the absence of DNA, but that lower-order structures form in the presence of their cognate
DNA (Boyle et al, 2009). To test whether DNA is a required cofactor for the formation of the TGA1-GRX480 complex, we developed a revised version of the pull-down assay, where TGA1 is bound to its cognate DNA sequence prior to incubation with an extract containing GRX480 (Supplemental Figure 8). We observed an interaction between TGA1 and GRX480 in this new DNA-based pull-down assay (Figure 8b lane 3). This conclusion can be reached from observing the presence of a band co-migrating with that found in the GRX480 input and its absence when an extract devoid of GRX480 is incubated with TGA1 bound to DNA (lane 4). Furthermore, GRX480 was not recovered from the pull-down when TGA1 was missing from the extract (lane 2). Next, we tested whether GRX480 could antagonize the enhanceosome activity of a TGA1-NPR1 complex using the in vivo transcription assay system (Figure 6). We found that the SA-dependent transcription activity of the TGA1-NPR1 enhanceosome was sabotaged by GRX480, which was observed by a significant decrease in transcription output of the enhanceosome in the presence of GRX480 (Figure 8c). In naïve cells, the enhanceosome did not form, consistent with a lack of interaction between the two proteins in these conditions. We also observed that, in the presence of SA, the TGA1-GRX480 complex did not activate transcription (Figure 8d), suggesting that the complex acted mainly as a passive repressor. However in naïve cells, there was a statistically significant difference between the output of TGA1 whether GRX480 was absent or present, which would suggest that the complex could potentially act as an active repressor. Regardless of the mode of repression, whether active or passive, the data support a model in which TGA1 and GRX480 form a transcriptionally repressive complex. The results of the transcription assay are consistent with the increased transcription output observed for the 23-gene set
when contained within the tga1/tga4 double mutant background and the grx480 RNAi line.

### 3.2.10 GRX480 is recruited to the PRI promoter via its association with TGA1/TGA4

The recruitment of several transcription regulators to the PRI promoter and their output is well established, both molecularly and genetically. Furthermore, their location at the PRI promoter and the availability and reliability of data regarding the feasibility of performing chromatin immunoprecipitation (ChIP) on this promoter is well recognized. This prior knowledge makes this gene a truly ideal system to study transcription control in immunity. The question then remains as to whether such TGA1/4-GRX480 complex could form at the PRI promoter. First, we investigated PRI’s capacity to recruit TGA1/4 by ChIP. We detected a constitutive recruitment in wild-type plants, with no significant difference between naïve and SA-treated cells. We only discerned background signals in the tga1/tga4 double mutant (Figure 8e). Next, we confirmed the role of TGA1/4 on PRI transcription by qRT-PCR. Consistent with the constitutive recruitment of TGA1/4 at the promoter, we observed an up-regulation of PRI in both the naïve and SA-treated cells of the tga1/tga4 double mutant (Figure 8f). Next, we examined whether GRX480 can be recruited to the PRI promoter and if the recruitment would also be constitutive, like that of TGA1/4. We found that GRX480 was present at the PRI promoter under naïve conditions, but that the recruitment was significantly enhanced by the treatment of cells with SA (Figure 8g). Consistent with this finding, we observed only an up-regulation of PRI in SA-treated cells of the grx480 RNAi line (Figure 8h). Finally, to test if the recruitment of GRX480 at the PRI promoter would require TGA1/4, we performed ChIP
against GRX480 in the *tga1/tga4* double mutant. We observed that in naïve and SA-treated cells, the GRX480 signal was significantly lower than in the wild-type plant (Figure 8i), confirming the recruitment of GRX480 by TGA1/4. Taken together, the results of this section indicate that a repressosome, composed of at least TGA1/4 and GRX480, assembles at the *PR1* promoter.

### 3.3 CONCLUSIONS

In this manuscript we demonstrate that TGA1 and TGA4 are genome-wide transcriptional repressors, but that they can nevertheless impart both positive or negative outputs to processes under their control. We have uncovered that TGA1/4 control a set of WRKY transcription factors involved in the negative regulation of basal immunity, which led to the discovery that clade I TGA are master-decoupling switches antagonizing the immune suppressive pole of the brassinosteroids-dependent growth-immunity tradeoff (Figure 9). This discovery positions TGA1/4 as prime targets for biotechnological, pharmacological or breeding approaches aimed at enhancing immunity without the usual cost to yield. We also demonstrate that TGA1/4 antagonizes the expression of NPR1-dependent SAR-genes through their recruitment, at promoters, of an unusual class of corepressors, the so-called novel glutaredoxins (GRX) (Figure 9). This class of GRX had only a speculated biochemical/enzymatic function and was without molecular function. However, we now provide evidence for their integration into repressosome complexes and thus shed light on the molecular function of this mysterious and large family of plant-specific proteins, which is involved in many important processes in addition to immunity.
3.4 DISCUSSION

Using a large-scale approach, we demonstrate that TGA1 and TGA4 are genome-wide transcriptional repressors, but that they can nevertheless impart both positive or negative outputs to processes under their control. In an attempt to understand the interrelationship between TGA1/4 and WRKY transcriptions in controlling basal immunity, we have uncovered an unprecedented function of the clade I TGA. We discovered that TGA1/4 feed into the well-known brassinosteroids-controlled growth-immunity tradeoff system to dampen the negative outcome played by this group of hormones on immunity. Previously, the prevailing thought was that the decision to grow or to mount an immune response is based on the allocation of limited resources and that a plant cannot grow and mount an immune response at the same time. However, because TGA1/4 only regulate the immune side of the tradeoff without affecting the growth side of it, clade I TGA1 are the linchpins providing the solution for decoupling this incessant tug-of-war. It is easy to understand why plants have evolved such growth-immunity tradeoff. In a natural environment, it is likely that individual plants from a population will be faced with the prospect of growing in a nutrition-poor soil. Since both growth and the deployment of immunity are energy intensive processes, growth is given priority for the purpose of setting seeds, with the caveat that on occasion resources are diverted towards fending off pathogens, also for the ultimate purpose of setting seeds. This tradeoff is built-in by design through the use of the same transcription factor, in this case BZR1, for the simultaneous stimulation of growth and suppression of immune responses. However, in an agricultural setting where it is possible to nutritionally-enrich the soil, this tradeoff is not only optional but is an undesirable trait. The entry-point of TGA1/4 in the tradeoff
at a position between BZR1 and its negative control on immunity, allows for the decoupling of the unilateral energy allocation towards growth or immunity. Furthermore, because TGA1/4 are hierarchically above a set of WRKYs controlling basal immunity, they can be aptly referred to as master-decoupling switches of this tradeoff system. Thus, because of their higher regulatory position, TGA1/4 reveal themselves as prime targets for biotechnological, pharmacological or breeding approaches aimed at enhancing immunity without the usual cost to yield.

3.5 METHODS

3.5.1 Plant Growth Conditions and Chemical treatments

Conditions for the growth of the wild-type Arabidopsis thaliana (Columbia), the tga1/tga4 double mutant and the GRX480-RNAi plants were as previously described (Despres et al., 2003). For RNA sequencing analysis, 4-5 week-old plants were treated with 300 µM SA for 4 h and immediately harvested and frozen in liquid nitrogen. Plants were stored at -80 °C until RNA isolation. For TGA1 and TGA4 expression analysis, 4-5 week-old plants were treated with 2.5 µM brassinazole (BRZ) in 0.016% v/v DMSO or mock-treated with 0.1% v/v DMSO for 24 h, after which they were harvested and stored in the same way as described above for RNA sequencing analysis.

3.5.2 Plant Transcription Assays and Plant Two-hybrid Assays

All procedures for the plant transcription and plant two-hybrid assays were performed as previously described (Boyle et al., 2009).

3.5.3 Chromatin immunoprecipitation (ChIP) assay

ChIP was performed as was described previously (Chakravarthy et al., 2003).

Two grams of leaf tissue was fixed for 30 min in 1% formaldehyde. Leaves were rinsed extensively with water, dried, frozen in liquid nitrogen, and stored at 80°C until further processing. Tissues were ground in IP buffer (0.1% SDS, 1% Triton X-100, 50 mM Hepes, pH 7.9, 150 mM NaCl, and 1 mM EDTA) supplemented with protease inhibitors. The lysate was centrifuged for 5 min at 14,000 rpm and 4°C in a microcentrifuge before filtering through siliconized glass wool. The chromatin was sonicated to yield DNA fragments between 0.5 and 1.3 kb in size. The chromatin solution
was split into two 500 µL aliquots and combined with 20 µL of protein A-Sepharose beads (Bio-Rad, Hercules, CA) that had been preadsorbed with 10 µL of TGA1/4 antibodies. The anti-TGA1/TGA4 antibody was described here (Shearer et al., 2012). After an overnight incubation at 4°C on a rotation wheel, the beads were washed three times with IP buffer and twice with TE, pH 8.0. The immunoprecipitated material was released from the beads by heating at 65°C for 15 min in 200 µL of TE supplemented with 1% SDS. After that, 400 µL of TE and 1% SDS was added to the suspension and incubated at 65°C for 6 h. After precipitation with isopropanol, sodium acetate, and 2 µg of glycogen, the DNA was resuspended in 40 µL of TE. Finally, qPCR was performed using the following primers. UBQ5 was used as the normalizing gene.

3.5.4 DNA-Based Pull-Down Assays

The biotinylated LS5-LS7 probe (5′-GTTCCTCTACGTCACTATTTACTTTACGTCATAGATGGG-3′-Biotin annealed to 5′-CCACATCTATGACGTAAGTAAAATAGTGACGAGAAAC-3′) was coupled to streptavidin iron oxide particles according to the manufacturer’s instructions (Sigma-Aldrich). His-tagged purified factor TGA1 in 500 µL of binding buffer (200 mM NaCl, 1 mM EDTA, and 20 mM HEPES, pH 7.9), was incubated with the beads for 30 min at room temperature, with continuous inversion. The beads were washed twice with 500 µL of binding buffer and once with binding buffer containing 1% milk. Crude *Escherichia coli*-produced GRX480, NPR1, a combination of GRX480 and NPR1, which were independently produced, or a crude *E. coli* extract in 500 µL of binding buffer, containing 1% (w/v) milk, was added to the beads containing the DNA-TGA1 complex and was incubated for 30 min. After this incubation, the beads were washed twice without milk as described above. Proteins were eluted by boiling in 45 mL of SDS-PAGE sample buffer and subjected to immunoblot analysis. Note that NPR1 contains a mutation in the BTB/POZ, which allows for higher expression, as explained previously.

3.5.5 Quantitative RT-PCR

Total RNA was extracted from leaves using the Aurum Total RNA Mini Kit (Bio-Rad) according to the supplier’s instructions. After treatment with DNase I (New England Biolabs), first strand cDNA synthesis was generated using iScript™ Reverse Transcription Supermix (Bio-Rad) according to the supplier’s instructions. Quantitative RT-PCR was performed in the same way as we do for qPCR (Wu et al., 2012). The sequences of the primers are as follow:
PR1F (5’-GCTCTTGTAGGTGCTTTGTTCTTCC-3’),
PR1R (5’-AGTCTGCAGTTGCCTTTAGTTGTC-3’),
WRKY40F (5’- ATCCACGACAAGTGCTTTGGTGA-3’),
WRKY40R (5’- GTTTCTTGAGGGGCTGTATT-3’),
GRX-qFw (5’-GGACGGAGAGGTGTGTC-3’),
RNAi-Long-Re (5’-CACAGAGCCCCAACTTC-3’),
UBQ5-1 (5’-ACCTACGTTTACCAGAAGAGGGAGTTGAA-3’),
UBQ5-2 (5’-AGCTTACAAAATTCACAATAGAATGCAG-3’),
AZF1a (5’- ATCTCGAGCAATTGGGCTAAA-3’),
AZF1b (5’- ATGAGGAGACAAAGAGCGAG-3’),
ATBCBa (5’- CTTGTATTGTGTTTGCTGC-3’),
ATBCBb (5’- AAGTTTTACCAGGCTAGCCCA-3’),
GRXS13.2a (5’- GGAAAATCTGATGGCTGCTC-3’),
GRXS13.2b (5’- AATCAAAGCCATAAGGCCCA-3’),
WRKY15-Fwd (5’- TCCTGTTGCTTCGCTGAAGA-3’),
WRKY15-Rv (5’- CTTATCGCCGGAACCCTAAT-3’),
WRKY48 fw (5’- GGTGTTCGTTTCTGACGAAGAGC-3’),
WRKY48 rv (5’- TGGTGAACCGTAATAGCCTCTGG-3’).

3.5.6 ROS quantification

Two 3.8mm diameter leaf disks were sampled per plant, one disk per leaf, from newly opened leaves. Leaf disks were floated on 150μL of either: MQH$_2$O (negative and positive controls), 0.016% DMSO (Brassinazole) or 0.028% DMSO (Bikinin) for mock treatments, or with 150μL of either: 2.5μM Brassinazole or 5μM Bikinin for 16 hours. Samples were induced for ROS production with 100μL of reaction mix (100μM of Luminol [Sigma-Aldrich], 100nM of FLG22 [Genscript] and 10μg of Horseradish Peroxidase [Sigma-Aldrich]). Luminescence was measured using a PolarStar OPTIMA unit for 40minutes, except in the negative and positive controls, which was 35minutes. Curves were integrated from 2minutes to 40minutes to construct the “Total Relative Luminescence Curves”.

3.6.7 RNA sequencing analysis

Total RNA was extracted as described above for Quantitative RT-PCR. 10 μg of total RNA were submitted to the Centre for the Analysis of Genome Evolution and Function (CAGEF) for library preparation and sequencing. Total RNA quality and quantity was analyzed using the Agilent BioAnalyzer (Agilent Technologies). mRNA was isolated from total RNA using Dynabeads mRNA purification kit (Thermo Fisher Scientific). The mRNA was sheared to 300 bp fragments on a focused-ultrasonicator (Covaris) and precipitated in ethanol. First and second strand cDNA synthesis was performed using cDNA synthesis kits from New England BioLabs. The cDNA libraries were then prepared using the NEBNext DNA library preparation kit (New England BioLabs) with in-house adapters. Adapter-ligated cDNA was size selected for 300 bp fragments using Ampure XP beads (Agencourt) and amplified following the PCR protocol described in the DNA library preparation protocol.
Proper size, quantity and quality of all sample libraries was checked as mentioned above. Libraries were pooled and loaded into an Illumina NextSeq500 sequencer at 1.8 pM final concentration. A 30% PhiX spike-in was added to the run to ensure optimal run performance. Sequencing was performed as single-end, unstranded reads (150 bp long). Raw sequence reads have been deposited in the National Center for Biotechnology Information Sequence Read Archive under the accession number SRP071011. Reads were first filtered to remove ribosomal RNA contamination using Bowtie2 with default settings (Langmead, B., & Salzberg, 2012; Quast et al., 2012). Clean reads were aligned to Arabidopsis thaliana genome (TAIR10) with TopHat2 (Kim et al., 2013). Mapped reads were used as input for Cuffdiff2 to estimate gene expression as FPKM (Fragments Per Kilobase of transcript per Million mapped reads) and identify differentially expressed genes ($q\text{-value} < 0.05$) (Trapnell et al., 2013).

3.5.8 Venn diagrams, Gene Ontology (GO) term analysis

Venny was used to generate Venn diagrams to study the degree of overlap between up- or down-regulated genes in each group analyzed ($q\text{-value} < 0.05$) (Oliveros and Venny). Gene Ontology (GO) map was generated using Cluego, a Cytoscape plugin (Bindea et al., 2009). The significance of each term was calculated using two sided hypergeometric test and $p$-value were corrected using the Bonferroni method. For the larger data set, GO term enrichment were selected when $p\text{-value} < 0.05$. GO terms are represented as nodes which are linked based on kappa score level ($\geq 0.3$). The node size represents the term enrichment significance. Only the most significant term in the group was labeled. The group color were randomly assigned by Cluego. Gene sets used to create the heatmaps were based on GO term analysis generated by the agriGO singular enrichment analysis tool (Du et al., 2010). The Arabidopsis genome locus TAIR10 was selected as reference and a Bonferroni multi-test adjustment method was applied to obtain the GO term enrichment with a $q\text{-value} < 0.05$. 
Figure 1. TGA1/4 positively regulate basal immunity by repressing negative regulators of the process.

a-b, Gene ontology map showing significantly over-represented GO terms in genes activated by TGA1/4 (a) and repressed by TGA1/4 (b). For a and b, GO term enrichment were selected when the p-values < 0.05. The node size represents the enrichment significance of the term. The most significant term in a group is assigned as group name. Group colors are randomly assigned by ClueGO.
Figure 2. TGA1/4 directly bind and repress a subset of WRKYs that negatively regulate basal immunity.

a-c, RT-qPCR using *WRKY15* (a), *WRKY40* (b) and *WRKY48* (c)-specific primers indicates the constitutive enhanced expression of the *WRKY* genes in the *tga1/tga4* double mutant versus wild-type. *Arabidopsis* leaves were untreated (white bars) or treated for 4 h with 300µM SA (grey bars). d-f, ChIP using an anti-TGA1/4 antibody performed in wild-type and *tga1/tga4* double mutant reveals a constitutive recruitment of TGA1/4 to the *WRKY15* (d), *WRKY40* (e) and *WRKY48* (f)-promoters. *Arabidopsis* leaves were untreated (white bars) or treated for 4 h with 300µM SA (grey bars). For a to f, the asterisks indicate significance (t test, *P < 0.05 and **P < 0.01). Data represent mean from biological triplicates ± 1SD.
Figure 3. The segment of basal immunity regulated by brassinosteroids is co-
controlled by TGA1/4

a, Venn diagram showing the number of genes coregulated by TGA1/4 and
brassinosteroids. The genes regulated by brassinosteroids are from Sun et al., 2010. b, Gene ontology map showing significantly over-represented GO terms in genes
coregulated by TGA1/4 and brassinosteroids. c, Gene ontology map showing
significantly over-represented GO terms in genes regulated by brassinosteroids only. For
b and c, GO term enrichment were selected when the p-values < 0.05. The node size
represents the enrichment significance of the term. The most significant term in the group
is assigned as group name. Group colors are randomly assigned by ClueGO.
Figure 4. TGA1/4 antagonize the brassinosteroid-dependent suppression of basal immunity

a, A model showing the brassinosteroids (BR) mediated growth-immunity tradeoff (black) and chemical agents used in this study to modulate the BR-pathway (red and green). b, Reduction of flg22-triggered ROS burst after bikinin treatment in wild-type plants. c, Enhancement of flg22-triggered ROS burst after brassinazole (BRZ) treatment in wild-type plants. d and e, Further reduction of flg22-triggered ROS burst after bikinin treatment in the tga1tga4 mutant plants compared with wild-type plants. f and g, Abolished enhancement of flg22-triggered ROS burst after brassinazole (BRZ) treatment in the tga1tga4 mutant plants compared with wild-type plants. For b to g, Flg22-triggered ROS bursts were measured for 40 min. For b, c, e and g, the total photon counts for each treatment during the 40 min. period were graphed. The asterisks indicate significance at p = 0.05. Data represent mean from 15 biological replicates ± 1SE. Of note, the mock conditions for brassinazole and bikinin experiments are different and hence are not expected to be the same (see methods).
Figure 5. TGA1/4 physically integrate into the growth-immunity tradeoff at WRKY promoters

**a and b**, RT-qPCR using WRKY15, WRKY40 and WRKY48-specific primers indicates a constitutive enhanced expression of the WRKY genes in the tga1/tga4 double mutant versus wild-type. *Arabidopsis* leaves were treated with mock solution (light blue bars, **a**) or treated with brassinazole (dark blue bars, **b**). **c-e**, ChIP using an anti-TGA1/4 antibody performed in wild-type and tga1/tga4 double mutant reveals the recruitment of TGA1/4 to the WRKY15 (**c**), WRKY40 (**d**) and WRKY48 (**e**) promoters after brassinazole treatment (dark blue bars). For a to e, the asterisks indicate significance (t test, *P < 0.05 and **P < 0.01). Data represent mean from biological triplicates ± 1SD.
Figure 6. A corepressor other than NPR1 is likely involved in the TGA1/4-repression of NPR1-regulated genes.

a, Scatter plots of gene expression levels (FPKM) revealing the proportion of up- (red) and down- (green) regulated genes in the tga1/tga4 double mutant compared to wild-type, both treated with SA for 4 h. b, Box-plot of all genes in (a), indicating that TGA1 is a global repressor. c, Scatter plots of the TGA1-regulated genes found in (a) that are coregulated with NPR1. Red and green indicate genes up- and down-regulated in the npr1 mutant, respectively. Kendall coefficient of correlation ($\tau_a$) is presented for genes that are up- and down-regulated in the tga1/tga4 double mutant. d, Box-plot of all genes in (c), indicating that TGA1 is a repressor of NPR1-regulated genes. e and f, TGA1 forms an enhanceosome in which the transcription activation properties are dependent on the transactivation domain of NPR1. e, A Gal4-UAS transcription system was used to reveal that TGA1 forms an enhanceosome with NPR1, whose function depends on cysteines 521 and 529. f, Control using a plant two-hybrid system showing that TGA1 interacts with wild-type NPR1 and a mutant harboring a cys-to-ser replacement at positions 521 and 529. For e and f, NS indicates nonsignificance at $p = 0.05$ and the asterisks indicate significance at $p = 0.05$. Data represent mean from biological triplicates $\pm$ 1SD. g, Model depicting the enhanceosome that is formed in the plant transcription assay. h, Model depicting the putative coregulator sabotaging the complex, which occurs in the endogenous system.
Figure 7. GRX480 is a transcription repressor of TGA1/4-dependent NPR1-regulated genes.

a, Potential TGA1/4 coregulators from the initial filtering and after filtering using keywords related to immunity (number in parentheses). b, Venn diagram showing the number of coregulated genes between the tga1/tga4 double mutant and the GRX480 RNAi line. Kendall coefficient of correlation ($\tau_a$) is presented for genes that are up- and down-regulated in the GRX480 RNAi line. c, Scatter plots of gene expression levels (FPKM) revealing the proportion of up- (red) and down- (green) regulated genes in the GRX480 RNAi line compared to wild-type, both treated with SA for 4 h. d, Box-plot of all genes in (c), indicating that GRX480 is a global repressor. e, Scatter plots of the GRX480-regulated genes found in (c) that are coregulated with NPR1. Red and green indicate genes up- and down-regulated in the npr1 mutant, respectively. Kendall coefficient of correlation ($\tau_a$) is presented for genes that are up- and down-regulated in the GRX480 RNAi line. f, Box-plot of all genes in (e), indicating that GRX480 is a repressor of NPR1-regulated genes. g, Venn diagram showing the number of coregulated genes between the tga1/tga4 double mutant, the GRX480 RNAi line and the npr1 mutant. h, Gene ontology map showing significantly over-represented GO terms in genes coregulated by TGA1/4, GRX480 and NPR1.
Figure 8. TGA1-GRX480 repressosome and its impact on NPR1-dependent processes.

**a**, Classic co-immunoprecipitation assay showing that TGA1 does not recruit GRX480 (black arrow) and that a cofactor is missing. **b**, DNA-based pull-down assay showing that TGA1 (white arrow) recruits GRX480 when bound to its cognate DNA. Lane 2 indicates that GRX480 is not recruited to the DNA in the absence of TGA1. **c,d**, A Gal4-UAS transcription system was used (**c**) to reveal that GRX480 sabotages the TGA1-NPR1 enhanceosome and to (**d**) show that the TGA1-GRX480 complex does not activate transcription. **e**, ChIP using an anti-TGA1/4 antibody performed in wild-type and tga1/tga4 double mutant reveals a constitutive recruitment of TGA1/4 to the PR1 promoter. **f**, RT-qPCR using PR1-specific primers indicates the enhanced SA-expression of the PR1 gene in the tga1/tga4 double mutant versus wild-type. **g**, ChIP, using an anti-GRX480 antibody (Supplemental Figure 9), performed in wild-type and GRX480 RNAi line reveals an SA-inducible recruitment of GRX480 to the PR1 promoter. **h**, RT-qPCR using PR1-specific primers indicates the enhanced SA-expression of the PR1 gene in the GRX480 RNAi line versus wild-type. **i**, ChIP using an anti-GRX480 antibody performed in wild-type and tga1/tga4 double mutant reveals a TGA1/4-dependent recruitment of GRX480 to the PR1 promoter. For d to j, NS indicates nonsignificance at p = 0.05 and the asterisks indicate significance at p = 0.05. Data represent mean from biological triplicates ± 1SD.
Figure 9. Immunity networks regulated by brassinosteroids, salicylic acid and TGA1/4.

In this study, we found that TGA1/4 antagonize the immune control of two major phytohormones, brassinosteroids (on the left) and salicylic acid (on the right) through distinct molecular mechanisms. TGA1/4 integrate into the brassinosteroid-mediated growth-immunity tradeoff through their direct control of WRKYs. Brassinosteroid-mediated suppression of immunity is dampened by the action of TGA1/4. We also found here that TGA1/4 and GRX480 can form a complex that interferes with the coactivator function of NPR1, affecting, to some degree, NPR1-dependent immunity.
Table 1. TGA1/4 regulated *WRKY* genes (q<0.01)

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<tr>
<th>TGA1/4 repressed <em>WRKY</em></th>
<th>Role in immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td>WRKY15</td>
<td>Negative regulator of basal immunity (Lozano-Durán et al., 2013)</td>
</tr>
<tr>
<td>WRKY40</td>
<td>Negative regulator of basal immunity (Xu et al., 2006)</td>
</tr>
<tr>
<td>WRKY48</td>
<td>Negative regulator of basal immunity (Xing et al., 2008)</td>
</tr>
<tr>
<td>WRKY22</td>
<td>Positive regulator of submergence-triggered immunity (Hsu et al., 2006)</td>
</tr>
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<td>WRKY33</td>
<td>Positive regulator of immunity against necrotrophic fungal pathogens (Zheng et al., 2006)</td>
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<td>WRKY46</td>
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<table>
<thead>
<tr>
<th>TGA1/4 activated <em>WRKY</em></th>
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<td>None</td>
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Supplemental Figure 1. Gene ontology map of TGA1/4-only subset showing that the regulation of immune processes by TGA1/4 is both brassinosteroid-dependent and independent.

Significantly over-represented GO terms in genes regulated by TGA1/4-only are shown. GO term enrichment were selected when p-value < 0.05. The node size represents the term enrichment significance. The most significant term in the group was assigned as group name. Group colors are randomly assigned by ClueGO.

Supplemental Figure 2. ROS quantification demonstrating that flg22 induces ROS production in wild-type plants.

- **a**, Flg22-triggered ROS bursts were measured for 35 min in wild-type plants.
- **b**, Total photon counts for each treatment during the 35 min. period were graphed.
Supplemental Figure 3. Hypocotyl length measurements indicating that brassinazole-treated tga1/tga4 mutant plants show normal restricted growth associated with a wild-type phenotype.

Hypocotyl lengths of 7-day-old seedlings grown in the dark were measured. bzr1-1D is a mutant insensitive to brassinazole and shows no significant changes in hypocotyl length after treatment.

Supplemental Figure 4. Venn diagram showing the number of coregulated genes between the tga1/tga4 double mutant and the npr1 mutant.

The microarray data for the NPR1 DEG is from published data (Shearer et al., 2012).
Supplemental Figure 5. Flow chart of the strategy used to identify transcription factor-coregulators.

In this study, we used the double mutant tga1/tga4, characterized previously (Shearer et al., 2012), to perform the first round of RNA-sequencing (Step 3).
Supplemental Figure 6. Characterization of the grx480 RNAi line selected for RNA-Seq.

a, RT-qPCR, using GRX480-specific primers, demonstrates the significant reduction of the GRX480 transcript in the GRX480 RNAi lines versus wild-type after a 4 h SA-treatment (last three bars). grx480 RNAi line #4 was used to perform RNA-seq experiments. No significant difference was detected prior to SA treatment (first three bars). The asterisks indicate significance at \( p = 0.05 \). Data represent mean from biological triplicates ± 1SD. 
b, Photographs of typical wild-type and GRX480 RNAi line plants showing an absence of visible phenotype.

Supplemental Figure 7. Verification of RNA-seq data in another grx480 RNAi line (line #6).

da-d, RT-qPCR, using AZF1 (a), ATBCB (b), GRXS13 (c) and PR1 (d)-specific primers, demonstrates the significant induction of the transcripts in the grx480 RNAi line #6 versus wild-type after a 4 h SA-treatment (grey bars). The asterisks indicate significance at \( p = 0.05 \). Data represent mean from biological triplicates ± 1SD.
Supplemental Figure 8. DNA-based pull-down assay of GRX480 by DNA-bound TGA1.

The biotinylated DNA molecule was double-stranded and contained the LS7 element of the PRI promoter (Despres et al., 2000), which harbors a TGA-transcription factor binding-site (Despres et al., 2000). The matrix was streptavidin-bound paramagnetic beads.

Supplemental Figure 9. Specificity of the anti-GRX480 antibody.

a, Peptide from the GRX480 protein used to generate the anti-GRX480 antibody. The C-terminal cysteine was for coupling to a carrier protein and is not part of the native sequence. b, Recombinant GRX480 and Roxy 2 proteins contained within a crude E. coli extract, were visualized by Ponceau staining after separation by SDS-PAGE and transfer to a nitrocellulose membrane prior to detection with the anti-GRX480 antibody. The antibody detects only GRX480. Roxy 2 (Xing et al., 2008) is another member of the GRX family.
Supplemental Table 1. Potential TGA1 and TGA4 coregulators.

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<tr>
<th>Tracking id</th>
<th>Gene short name</th>
<th>Annotation</th>
<th>q value</th>
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<td>AT1G28480</td>
<td>GRX480</td>
<td>Protein disulfide oxidoreductase</td>
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<td>Kinase</td>
<td>0.00179169</td>
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<td>AT2G30020</td>
<td>Phosphatase</td>
<td>0.00179169</td>
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<td>Phosphatase</td>
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<td>ATMKP5</td>
<td>Kinase</td>
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<td>AT1G03850</td>
<td>GRXS13</td>
<td>Protein disulfide oxidoreductase</td>
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**Supplemental Table 2. Importance of the 23 genes coregulated by TGA1/4, GRX480 and NPR1.**

<table>
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<th>Tracking id</th>
<th>Gene name</th>
<th>Importance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G03850</td>
<td>GRXS13</td>
<td>Negative regulator of <em>Botrytis cinerea</em> infection.</td>
<td>(La Camera et al, 2011)</td>
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<tr>
<td>AT1G10340</td>
<td>AT1G10340</td>
<td>NPR1-dependent and BTH responsive gene.</td>
<td>(Blanco et al, 2009)</td>
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<tr>
<td>AT1G24140</td>
<td>AT1G24140</td>
<td>Transcription significantly induced by toxic chemical, 2,4,6-trinitrotoluene (TNT).</td>
<td>(Gandia-Herrero et al, 2008)</td>
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<tr>
<td>AT1G32920</td>
<td>AT1G32920</td>
<td>Expression is rapidly induced by wounding.</td>
<td>(Walley et al, 2007)</td>
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<tr>
<td>AT1G66090</td>
<td>AT1G66090</td>
<td>Hypothesized to play an important role in plant innate immune system.</td>
<td>(Meyers et al, 2002)</td>
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<tr>
<td>AT2G14610</td>
<td>PR1</td>
<td>Marker gene of systemic acquired resistance.</td>
<td>(Ryals et al, 1996)</td>
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<tr>
<td>AT2G18690</td>
<td>AT2G18690</td>
<td>Transcription significantly induced by aphid infestation.</td>
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<td>AT2G27660</td>
<td>AT2G27660</td>
<td>Hypothesized to be involved in plant response to salt stress.</td>
<td>(Gao et al, 2016)</td>
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<td>AT2G40140</td>
<td>CZF1</td>
<td>Cold-induced transcription factor.</td>
<td>(Vogel et al, 2005)</td>
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<td>AT3G01830</td>
<td>CM140</td>
<td>Transcription is induced by ozone and cycloheximide treatment.</td>
<td>(McCormack et al, 2005; Zhou et al, 2015)</td>
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<td>AT3G02840</td>
<td>AT3G02840</td>
<td>Transcription responds immediately to fungal elicitation.</td>
<td>(Balazadeh et al, 2012)</td>
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<td>AT3G10930</td>
<td>Transcription significantly induced by selenate.</td>
<td>(Hoewyk et al, 2008)</td>
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<td>AT3G22910</td>
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<td>Expression is strongly stimulated by bacteria.</td>
<td>(Banza et al, 2016)</td>
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<td>AT3G28340</td>
<td>GATL10</td>
<td>Transcription induced by flg22 and surfactant treatment.</td>
<td>(Madhou et al, 2005; Navarro et al, 2004)</td>
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<tr>
<td>AT3G50930</td>
<td>BCS1</td>
<td>Stress responsive gene. Encodes a protein involved in the assembly of cytochrome bc1.</td>
<td>(Ho et al, 2008)</td>
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<td>AT3G61190</td>
<td>BAP1</td>
<td>Negative regulator of defense response.</td>
<td>(Yang et al, 2006)</td>
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<tr>
<td>AT4G39670</td>
<td>AT4G39670</td>
<td>Defense gene involved in effector-triggered immunity (ETI).</td>
<td>(DeFraia et al, 2010)</td>
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<tr>
<td>AT5G20230</td>
<td>ATBCB</td>
<td>Involved in lignin synthesis.</td>
<td>(Ezaki et al, 2005)</td>
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<tr>
<td>AT5G47850</td>
<td>CCR4</td>
<td>Essential for RNA editing in chloroplasts.</td>
<td>(Kotera et al, 2005)</td>
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<td>AT5G54490</td>
<td>PBP1</td>
<td>Affects the activation of its binding partner PYK10, a β-glucosidase, in damaged tissue.</td>
<td>(Nagano et al, 2005)</td>
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<td>AT5G67450</td>
<td>AZF1</td>
<td>Transcription induced by PAMP.</td>
<td>(Thilmony et al, 2006)</td>
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</table>

Genes highlighted in bold encode known important regulators of plant immunity. SAR marker gene, *PRI* is highlighted in red.
3.6 REFERENCE


Rochon A, Boyle P, Wignes T, Fobert PR, Despres C. 2006. The coactivator function of Arabidopsis NPR1 requires the core of its BTB/POZ domain and the oxidation of C-


CHAPTER 4 - Combining fungicides and prospective NPR1-based “just-in-time” immunomodulating chemistries for crop protection.

Contributions

I initiated, designed and performed all the experiments involved in this manuscript under the guidance of Dr. Després. I wrote the manuscript with the help of all the other authors.

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Xiahezi Kuai, Charles Barraco and Charles Després

Department of Biological Sciences, Brock University, St. Catharines, ON, Canada

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4.1 ABSTRACT

Each year, crop yield is lost to weeds competing for resources, insect herbivory and diseases caused by pathogens. To thwart these insults and preserve yield security and a high quality of traits, conventional agriculture makes use of improved cultivars combined with fertilizer and agrochemical applications. However, given that regulatory bodies and consumers are demanding environmentally safer agrochemicals, while at the same time resistance to agrochemicals is mounting, it is crucial to adopt a “holistic” approach to agriculture by not excluding any number of management tools at our disposal. One such tool includes chemicals that stimulate plant immunity. The development of this particular type of alternative crop protection strategy has been of great interest to us. We have approached this paradigm by studying plant immunity, specifically systemic acquired resistance (SAR). The deployment of SAR immunity requires the production by the crop plant of an endogenous small molecule metabolite called salicylic acid (SA). Furthermore, immunity can only be deployed if SA can bind to its receptor and activate the genes responsible for the SAR program. The key receptor for SAR is a transcription coactivator called NPR1. Since discovering this NPR1-SA receptor-ligand pair, we have embarked on a journey to develop novel chemistries capable of deploying SAR in the field. The journey begins with the development of a scalable assay to identify these novel chemistries. One such assay, presented here, is based on differential scanning fluorimetry technology and demonstrates that NPR1 is destabilized by binding to SA.
4.2 INTRODUCTION

A compelling analogy for agricultural systems is that of the simple gravity pendulum (Figure 1). Prior to the domestication of plants and any major human interventions on the planet, plant-pathogen interactions in the natural environment would have been at equilibrium or what could be described as the “normal” state. In some years or under certain conditions, the pathogen population may be more prevalent, while in some other instances, it is the host population that would have been more prevalent. These swings were likely never far from equilibrium since the goal of the pathogen population is to “nibble” at the host just enough to ensure that its reproductive cycle is completed, without wiping the host population out of existence. For the host population, its goal is to expend just enough energy to fight the pathogen so that it can complete its own life cycle and set seeds for the next generation. Under this paradigm, a plant population can allow a substantial amount of “yield” loss to pathogen attack as long as the capacity to reproduce is met. Furthermore, along with yield loss, pathogen infections often produce toxic chemicals incompatible with human consumption. This paradigm clearly clashes with human needs and as such, we have invented our agriculture with the goal of moving and holding the pendulum away from pathogen prevalence to maximize plant yield and quality. In human terms, moving and holding the pendulum high above our head requires a constant expense of energy. In agricultural terms, holding the pendulum is achieved through proper agricultural practices, the usage of elite cultivars and the treatment of crops with fertilizers and chemicals such as pesticides. Removal of the forces holding the pendulum would quickly lead to the system reverting to equilibrium or “normality”.

In the developed world, food is plentiful, relatively inexpensive and of high quality. Complacency has set in and made us forget that such abundance does not represent “normality” within the context of human history. In fact, scarcity is the norm and the profusion of high quality food is exceptional and a relatively recent phenomenon, which still has not reached all corners of our planet (Godfray et al., 2010). Since the dawn of agriculture, humans have been battling the elements and fighting crop diseases and weed infestations to safeguard their crops and ensure their own survival. Crop protection is a serious matter and is a constant battle against the tremendous force exerted by a system wanting to return to its equilibrium point, its “normality”. Fungal and bacterial pathogens represent part of this formidable force and are therefore one of the major causes of crop loss worldwide (Oerke and Dehne, 2004). Despite the sophisticated crop protection practices of today, the average yield lost to fungal and bacterial pathogens still hovers around 11% for barley, cottonseed, maize, oilseed rape, potatoes, rice, soybean, cotton, sugar beet, tomatoes and wheat (numbers from 2001-2003; Oerke, 2006). All means that can assist in keeping the pendulum high and away from its natural equilibrium point should be evaluated and potentially developed. In this perspective, we will discuss current countermeasures used to preserve crop yield security and quality threatened by pathogens, specifically fungi. We will also discuss the development of novel crop protection chemistries modulating plant immunity centered on the identification of new agonists of the salicylic acid (SA) receptor NPR1.

4.2.1 Fungicides: A key turning point in the war against pathogenic crop loss

The application, on a cultivated field, of crop protectants, which are generally grouped under the umbrella-term “pesticides”, plays a significant role in safeguarding
crop productivity. When the pathogens to combat are fungi, special pesticides termed fungicides are applied to the crop. Fungicides are chemical agents that, by definition, kill fungi or fungal spores (Haverkate et al., 1969). Humans have been using natural antifungal agents to control plants disease long before they realized that fungi were a causative agent of agricultural damage. Dating back to around 60 AD, wine was already used in cereal seed treatment to prevent disease (Russell, 2005). Interests in controlling fungal disease rose greatly after the devastating Irish Potato Famine of the mid-nineteenth century, which resulted in the death of approximately one million people (Gráda and Eiríksson, 2006). The Famine was caused by a fungal disease, called potato late blight. In 1885, the Bordeaux mixture, the first fungicide to be widely used worldwide, was serendipitously discovered by French botanist, Pierre-Marie-Alexis Millardet (Millardet et al., 1933). The Bordeaux mixture, a bitter tasting concoction prepared by mixing copper sulfate (CuSO₄) and slaked lime [Ca(OH)₂], was initially used on vines near roadways in order to discourage thieves from stealing grapes. Millardet discovered that downy mildew was less abundant on vines treated with the mixture. This accidental discovery turned out to be a very effective means to control many other diseases and for example, a twenty years study conducted in Vermont, US (1890-1910) showed that the control of potato blight with Bordeaux mixture increased the average yield of the potato crop by 64% (L. Jones et al., 1912). Up to the early 1900s, agricultural fungicides were mainly homemade by farmers using active ingredients such as sulphur, lime and copper sulphate. However, the 1930’s brought a radical change to agriculture with the development of the first synthetic chemical fungicides (nabam, thiram and zineb) (Gianessi and Reigner, 2005). Yields from potato crops treated with zineb and nabam
were 23-35% higher than those obtained by spraying with the Bordeaux mixture (Muncie and Morofsky, 1947; Wilson and Sleesman, 1951). Because of the superior performance of synthetic fungicides, fungicide production rapidly shifted from “do-it-yourself” concoctions to commercially produced chemistries. This paradigm shift signaled the beginning of the crop protection industry as we know it today. To date, some 110 different groups of fungicide with at least 10 different modes-of-actions have been discovered (Source: Fungicide Resistance Action Committee). In modern agriculture, synthetic fungicides, without doubt, play a significant role in safeguarding crop quality and yield. Typically, a synthetic fungicide recommended for a specific crop-fungus couple will provide 90% or greater control of the target disease (Gianessi and Reigner, 2005). Without their protection, it is estimated that the yield of most fruit and vegetable crops would fall by 50-95% due to plant disease (Gianessi and Reigner, 2005).

Unfortunately, despite the effectiveness of fungicide for yield security and the production of toxin-free crops, pathogens respond to the use of these chemicals by developing resistance. Fungicide resistance is said to have occurred when a chemistry with a specific mode-of-action has lost its ability to kill or inhibit fungal growth (Brent and Hollomon, 1995). This natural phenomenon is akin to antibiotics resistance in the treatment of human diseases of bacterial origin. Not unlike the population of the human species, the population of a specific fungal species consists of genetically diverse individuals. Within a fungal population, there are very rare individuals that, by chance, have just the right genetic make-up to survive the normally lethal effects of a fungicide. These are said to be individuals resistant to the fungicide. When a fungicide is used for many seasons to protect a crop, the descendants of these rare resistant individuals are the
only ones that can survive the treatment and as a result become the more dominant constituents of the population. As the percentage of resistant fungal individuals increases in the population, the effectiveness of the fungicide decreases to the point where it becomes completely ineffective and/or not economically viable to use as a crop protectant. It is also observed that the more effective a fungicide is at killing the targeted fungus, the higher the selective pressure is on the fungal population and the faster the fungicide resistance develops (Brent and Hollomon, 1995). The first instance of fungicide resistance in the field was observed in 1960 (Eckert, 1982). Up to the 1970s, there were only a few severe cases of fungicide resistance and the time taken for resistance to emerge was relatively long, ranging from 10 to 40 years. However, since the 1970s, the incidence of resistance has increased dramatically. Today, all major groups of fungicides have a reduced breadth of efficacy due to severe cases of resistance in certain fungal populations (Brent and Hollomon, 1995). Furthermore, the elapsed time before resistance emerges is often rather short (under 10 years). For example, in the case of the strobilurin (aka Quinone outside Inhibitors or QoIs) class of fungicides, which is profusely used because of its activity against all major fungal genera (Heaney et al., 2000), the first case of resistance developed after only two years following commercial introduction of the product. The emergence of fungal pathogen resistance to fungicide has become a widespread and severe problem in agriculture, which threatens yield security and crop quality. Alarmingly, food production is not the only way fungicide resistance affects humans. Resistant fungal species are also threatening human health, with the root-cause potentially stemming from agricultural practices.

Typically, when a human is afflicted by a disease, metabolic or pathogenic in
origin, it is likely that this person will take medication, which could be, for example, delivered in the form of a pill (oral administration) or intravenously. These routes of administration are designed to provide a specific and precise dosage (an amount of drug/kg body weight) and to confine the drug to the individual afflicted by the disease, without the drug reaching, for example, family members living under the same roof. Contrary to the treatment of human disease, the principal method of fungicide delivery in agriculture is through spraying (Brent and Hollomon, 1995). This application method provides a specific and precise dosage (an amount of fungicide/surface area of cultivated field). However, it does not confine the “medication” only to individuals, but it also “treats” the space between them. As such, spraying requires significantly higher amounts of “medication” for similar therapeutic effects as would be observed in humans. Critically, certain fungicide classes, like Azoles, are utilized to combat both human and crop fungal infections, increasing the probability for resistance to develop. It is thought that the massive use of azoles in agriculture has resulted in the emergence of multi-azole-resistant *Aspergillus fumigatus* isolates, the fungal agent responsible for invasive aspergillosis in human (Verweij et al., 2009). The group also pointed out that in 2004, the volume of azoles and azole-like agricultural fungicides used in the Netherlands was about 320-times higher than that of azoles used in clinical medicine in the vicinity of 130000 kg vs 400 kg. The large concentrations of applied azoles in agriculture, more than likely, lead to the accelerated evolution of *A. fumigatus* species for azole-resistance giving rise to aspergillosis in humans (Verweij et al., 2009). Such examples of fungicide resistance give ample reasons to look for means to minimize the use of fungicides and embrace a more “holistic” approach to crop protection. The bob of the pendulum is very heavy and
recruiting more people to hold it above head reduces the individual effort required to maintain the status quo. The corollary is that under this paradigm, the status quo is not jeopardized should one individual pass away. The pendulum analogy stresses that it is riskier to rely heavily on a single strategy, fungicides, as the pillar of fungal crop protection. Catastrophic crop failures could arise, should certain fungicide classes die on us as a consequence of being ineffective due to fungal resistance.

Despite the large number of fungicide chemical structures developed for agriculture, the target disease in each major crop-fungus couple can only be controlled by three or four different classes of fungicides (Brent and Hollomon, 1995). Current fungicide resistance management strategies include applying a mixture of fungicides with different modes-of-action and following strict guidelines for application regimen and concentrations. In parallel, efforts are made to discover new fungicides with multi-site mode-of-actions (Brent and Hollomon, 1995). These agrochemical management strategies cannot prevent the emergence of fungicide resistance, especially not those relating to human diseases, but they have been shown to be effective at delaying fungal resistance relating to crops.

4.2.2 “Just-in-time” immunomodulating chemistries for crop protection

In human medicine, the use of drugs that kill pathogens, such as fungicides and antibiotics, is not the only solution available to combat microbes. Alternative approaches that rely on boosting the immune system, such as immunization, are an important line of defense against pathogens. The immune systems of plants and animals operate quite differently, but it is nevertheless possible to develop strategies that can boost plant immunity. However, in plants there exist a tradeoff between immunity and
growth/development and many attempts at engineering constitutive immunity results in plants with suboptimal growth and development profiles, which affects yield directly (Heil and Baldwin, 2002). This tradeoff can be mitigated to a large extent by inducing immunity at the appropriate time, which can be accomplished by treatments with chemicals that boost immunity on demand and only when needed. This “just-in-time” philosophy was pioneered by Toyota for their Production System. Supplying "what is needed, when it is needed, and in the amount needed" according to their production plan is an important reason for Toyota’s success in the fierce automobile manufacturing market. For Toyota, the “just-in-time” strategy can “eliminate waste, inconsistencies, and unreasonable requirements, resulting in improved productivity”. By analogy, for crops,triggering immunity “just-in-time” would allow for optimum allocation of limited resources to resistance, when needed and for the amount of time needed, and resumption of growth and development once a threat has been neutralized, minimizing yield loss. As an added benefit, since these immunomodulators will target the crop and not the pathogen, the rate at which resistance manifests itself should be virtually nil, if properly implemented. Furthermore, immunomodulators that target NPR1, in principle, could be used across multiple plant species as NPR1 is found and is conserved in all crops of commercial significance (Kuai and Després, 2016). Recent advances in our understanding of the molecular mechanisms of plant immune responses, specifically those regulated by NPR1, have provided us with an opportunity to develop new agrochemicals through target-based pharmaceutical-style approaches, which we will discuss in the following sections.
4.2.3 The SA-receptor NPR1 as a validated immunomodulating target

Plants have evolved a variety of perception systems to recognize initial attacks from pathogens (Thomma et al., 2011). These systems are often referred to as microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs)-triggered immunity and effector triggered immunity (J. D. Jones and Dangl, 2006). After perceiving the initial threat, plants establish a broad-spectrum and long-lasting resistance, coined systemic acquired resistance (SAR), to protect themselves from the pathogenic invaders (Ryals et al., 1996). Another type of resistance, induced by beneficial microbes, termed induced systemic resistance, also exists and is reviewed elsewhere (Pieterse et al., 2014). SAR can be induced by avirulent pathogens and can confer resistance to a wide range of normally virulent pathogens, including bacterial, viral and fungal pathogens. This SAR response is mediated by the phytohormone, salicylic acid (SA) (Vlot et al., 2009). SA controls plant defense responses through its receptor protein, NPR1. Upon binding with SA, NPR1 undergoes a conformational change allowing it to act as a transcriptional coactivator and to activate the transcription of pathogenesis-related genes, which play a role in plant immunity (Ward et al., 1991; Wu et al., 2012). Although several hormones are involved in modulating SAR (Pieterse et al., 2009), the presence of SA and the NPR1 protein are two absolute requirements for the induction of SAR in plants. Mutant plants that cannot accumulate normal concentration of SA, or with mutations in NPR1 fail to establish SAR in response to pathogen challenge (Cao et al., 1997; K. Lawton et al., 1995). Exogenous application of SA can replace an initial avirulent pathogen challenge and stimulate a plant’s immune responses (White, 1979), which brought to light the feasibility of using chemicals to activate plant immunity on demand. Although SA could, in theory, be used
directly as an agrochemical, its application is limited by its chemical stability and rapid catabolic inactivation (Uknes et al., 1993).

The purpose of developing NPR1 agonists, that could deliver a “just-in-time” boost to crop immune system, is not meant to replace the current effective fungicides. The objective is to complement the use of fungicides in a comprehensive anti-resistance strategy to extend their commercial life and to ensure long-term global food security. Since SAR can provide broad-spectrum resistance, prospective NPR1 agonists could also prove effective in controlling bacterial and viral pathogens.

The chemical stimulation of crop immune responses is already successful in controlling pathogens. Benzo (1,2,3)-thiadiazole-7-carbothiolic acid (BTH) was first discovered as an inducer of SAR in 1996 and later brought to market by Syngenta as the active ingredient in a commercial product, Actigard (Bion in Europe). BTH was believed to act at the site or downstream of SA induced defense response pathway because BTH can induce resistance in a mutant plant that cannot accumulate normal concentrations of SA (K. A. Lawton et al., 1996). In 2012, we have demonstrated that BTH interacts with the SA-receptor, NPR1, with similar or a slightly better binding affinity than SA (Wu et al., 2012). Another example is Bayer CropScience’s recently introduced Isotianil, which protects rice crops from fungal infection by inducing SAR (Ogawa et al., 2011). However, these agrochemicals suffer from limited crop range. It is true that BTH can be used on 122 crop plants in the USA (Syngenta USA label on Actigard 50WG), dosage and registered crops vary by country. For example, in Canada, Actigard is only registered for use on tomato and tobacco. Furthermore, BTH cannot be used on the major crops (wheat, corn, potatoes, soybean, sugarcane and rice), while isotianil is only effective on
rice. Nonetheless, the existence of chemicals boosting crop immunity demonstrates the validity of developing such chemistries as crop protectants. Furthermore, both BTH and Isotianil have been developed without prior knowledge of their receptors. However, the fact, that NPR1 is the receptor for BTH, validates its suitability as a platform to develop new agrochemicals through target-based pharmaceutical-style approaches and raises the perspective that better chemistries with efficacies on a broad range of crops could be designed.

4.3 RESULTS

4.3.1 Advancing NPR1 to a druggable target: Differential Scanning Fluorimetry technology

Drug discovery assays are grouped into two broad categories; cell-based and target-based assays. The former is based on phenotype and includes chemical genetics, which has been used to identify molecules that modulate plant defense response (Noutoshi et al., 2012; Knoth et al., 2009). Target-based assays rely on an isolated protein, typically a receptor, to identify novel ligands, both agonists and antagonists. Given that we have identified a receptor-ligand couple in the form of NPR1-SA, the choice of using a target-based approach for drug discovery seemed logical for us. We have previously shown that NPR1 binds SA using equilibrium dialysis and scintillation proximity assays. Although these methods can be implemented for high throughput assay screens, they are cumbersome and make use of radioligands. In search of a simple, equilibrium based and non-radioactive assay, we opted to focus on thermodynamic methods. These are based on the change of protein thermal stability upon binding to a
ligand. In an equilibrium thermodynamics system, a protein population exists in one of two states, the native state (N) and the unfolded state (U) (Adkins, 1983).

If heat is added at a constant rate to this protein population system, the protein molecules will undergo conformational changes gradually shifting the equilibrium towards the unfolded state until every molecule is unfolded. Graphically, this transition from the native to unfolded state, as a function of temperature, appears as depicted in Figure 2a. The temperature required to reach the midpoint of this thermal transition, when the concentration of the native and unfolded forms of the protein are equal, is defined as the Melting Temperature ($T_m$). The $T_m$ is considered a good indication of protein stability, whereby a higher $T_m$ is indicative of a higher protein stability. Since ligand binding is known to affect protein stability, a shift in $T_m$ for a given protein can be observed after ligand binding (Cimmperman et al., 2008). One classic method used to measure the $T_m$ of protein is Differential Scanning Calorimetry (DSC) (Dassie et al., 2005). In a DSC experiment, the heat absorption of a protein population in a thermally-induced transition process is measured. DSC is a very accurate and direct way to measure protein $T_m$ and other thermodynamic parameters, such as, calorimetric denaturation enthalpy ($\Delta H$). However, this technique is rather low throughput, only one sample can be run at one time and each run typically takes one and half-hour per sample based on our experience. Another method that can be used to monitor protein $T_m$ is Differential Scanning Fluorimetry (DSF), also known as thermal shift or ThermoFluor (Niesen et al., 2007). A fluorescent dye that nonspecifically binds with hydrophobic regions of proteins is used during the course of a thermally-induced transition process. The fluorescence of the dye is quenched in aqueous solution but is very high in hydrophobic environments. As protein
unfolds, their hydrophobic regions are exposed and bind the dye resulting in an increase in the fluorescence signal. The fluorescence signal emitted by the dye is used to monitor the unfolding process of the protein. In a DSF experiment, the fluorescence intensity can be plotted as a function of temperature. The $T_m$ values can be calculated simply by determining the maximum of the first derivative. Thermal shift experiments can be done using conventional Real-Time PCR machine and are easily scaled up to 96 or 384 reactions assay. Therefore, it is widely used, in both academia and the pharmaceutical industry, in screens aimed at determining the best conditions for the stability of a given protein and in the early stages of drug discovery (Niesen et al., 2007). Many publications have successfully demonstrated the use of thermal shift in high throughput drug screening (Lo et al., 2004; Vedadi et al., 2006).

The thermal shift assay is therefore an obvious choice for the preliminary screening of small ligands that bind to NPR1. To develop a high-throughput screening assay, we must first ensure that NPR1 can bind SA in a thermal shift assay. We tested the C-terminal SA-binding domain of NPR1, Δ513, because of its better folding and higher production yield in *E.coli* protein expression systems. Upon SA binding, the $T_m$ of Δ513 shifted from 57°C to 54.3°C (Figure 2B and C). This result shows again the binding of SA to NPR1, bringing the number of assays, that have been used to demonstrate NPR1’s binding to SA, to six (Manohar et al., 2014; Wu et al., 2012). In addition, the data provide new structural insights on the nature of the NPR1-SA complex. The observation, that the $T_m$ of Δ513 was decreased by 2.6°C upon SA binding, indicates that SA destabilizes Δ513. Given that a protein population exists in two states during a thermal transition process, the native and unfolded states (equation 1), there are two possible explanations
for why SA destabilizes ΔS13. First, SA binds to the unfolded state of ΔS13 favoring the unfolding direction of the two-state process, which causes a decrease in $T_m$.

\[ N_{\Delta S13} \leftrightarrow U_{\Delta S13} \] (1)

\[ N_{\Delta S13} + SA \leftrightarrow U_{\Delta S13} \cdot SA \] (2)

Second, SA binds to the native state which causes the structure of ΔS13 to change to a less stable state (equation 3) before unfolding (equation 4), which results in a decrease in $T_m$.

\[ N_{\Delta S13} + SA \leftrightarrow \text{Lower-stability}_{\Delta S13} \cdot SA \] (3)

\[ \text{Lower-stability}_{\Delta S13} \cdot SA \leftrightarrow U_{\Delta S13} + SA \] (4)

Finally, once the assay is fine-tuned, the process for turning any assay into a high throughput screen (the scale up) consists in taking the single-tube assay, in our case the method to detect the NPR1-SA interaction, and to perform it simultaneously in a high multiple, for example in a 96-well plate. However, instead of using the same ligand in every well, in our case SA, a chemical library of diversified structures will be used allowing the interrogation of many potential ligands at one time. Chemicals that show a positive signal in an assay are called hits and are then subjected to further rounds of confirmation and analyses.
4.4 CONCLUSION

Advanced agricultural practices, classical breeding and crop protectants, dominated by fungicides, have been very effective at holding the pendulum away from pathogen prevalence. These “pillars” of modern agriculture have collectively ensured crop productivity and quality for contemporary human civilizations and they will likely continue to play a role in the future. However, the fungicide pillar is under attack and our capacity to hold the pendulum above our head is undermined. We propose to exploit NPR1 as a druggable target to develop “just-in-time” immunomodulating chemistries for crop protection as part of a “holistic” approach to extend the commercial life of effective fungicides and ensure future crop yield security. Plant immunomodulators should show no resistance from pathogens and could be used in an alternate regimen with fungicides or in combination with sublethal doses of fungicides. The SA receptor, NPR1, is a validated target and the differential scanning fluorimetry technology appears to be promising for the implementation of a target-based pharmaceutical-style high throughput screening platform to develop agonists for next generation crop protectants.
4.5 METHODS

4.5.1 Purification of recombinant proteins

Proteins were expressed in *E. coli* as N-terminal fusions to the 6xHIS tag according to standard protocols. Recombinant proteins were purified using HisTrap columns (GE) according to the manufacturer’s protocol. The binding buffer contained 20 mM sodium phosphate at pH 7.2, 40 mM imidazole and 500 mM NaCl. Bound proteins were eluted in the same buffer supplemented with 500 mM imidazole. Proteins were desalted/buffer-exchanged right after purification using MiniTrap columns (GE) according to the manufacturer’s instructions.

4.5.2 Thermal shift assay

One (1) µg of recombinant Δ513 at final concentration of 0.05 µg/µl was used in the thermal shift assays. The reaction buffer consisted of 20 mM sodium phosphate, pH 7.2 and 5× SYPRO Orange dye (Sigma, S5692). The assays were carried with or without 0.03µM salicylic acid. The final reaction volume is 20 µl. Reactions were loaded in Multiplate (Biorad, MLL9601). Thermal shift assays were performed on a CFX96 spectrofluorometric thermal cycler (BioRad) at a scan rate of 1 °C/min.
Figure 1. Pendulum analogy of agricultural systems.

See text for more details. The little human represents all the non-natural inputs and technologies used to ensure crop yield and quality.
Figure 2. SA destabilizes the C-terminus of the NPR1 Δ513.

(A) Graphic of a typical thermally-induced protein unfolding in a DSF experiment. The $T_m$ is the value at the inflection point, which can be measured more precisely by determining the first derivative. It is typical to observe a loss of fluorescence once proteins are unfolded due to their aggregation. (B) First derivation melting curves of Δ513 in the absence (blue) or presence (red) of SA. The $T_m$ is the value at the maximum, which can be measured more precisely by determining the second derivative. (C) Melting temperature, where $T_m$ is the value of $x$ when $f''(x) = 0$, of Δ513 in the absence (blue) or presence (red) of SA. S.D. calculated from three biological replicates. Note that the S.D. from the No SA control (blue) is smaller than the data point symbol and hence does not show.
4.6 REFERENCE


Knoth, C., Salus, M. S., Girke, T., & Eulgem, T. (2009). The synthetic elicitor 3,5-
dichloroanthranilic acid induces NPR1-dependent and NPR1-independent mechanisms of


CHAPTER 5 - Repression of Lateral Organ Boundary Genes by PENNYWISE and POUND-FOOLISH Is Essential for Meristem Maintenance and Flowering in Arabidopsis

Contributions
This manuscript was the product of a collaboration-project with the lab of Dr. Shelley R. Hepworth at the University of Carleton. I was invited to participate in this project due to my expertise with ChIP techniques. My contributions to this document include Figure 5: J and I, which directly lead to the conclusion that ATH1 is a direct target of BOP1. I was directly involved in developing and testing hypotheses that were critical to the manuscript. I also contributed to the editing of the entire manuscript.

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Madiha Khan, Laura Ragni, Paul Tabb, Brenda C. Salasini, Steven Chatfield, Raju Datla, John Lock, Xiahezi Kuai, Charles Després, Marcel Proveniers, Cao Yongguo, Daoquan Xiang, Halima Morin, Jean-Pierre Rullière, Sylvie Citerne, Shelley R. Hepworth, and Véronique Pautot

Department of Biology, Carleton University, Ottawa, Ontario, Canada K1S 5B6 (M.K., P.T., B.C.S., S.Ch., J.L., S.R.H.); Institut Jean-Pierre Bourgin, Unité Mixte de Recherche 1318 Institut National de la Recherche Agronomique-AgroParisTech, Bâtiment 2, Institut National de la Recherche Agronomique Centre de Versailles-Grignon, 78026 Versailles cedex, France (L.R., H.M., J.-P.R., S.Ci., V.P.); Plant Biotechnology Institute, National Research Council Canada, Saskatoon, Saskatchewan, Canada S7N 0W9 (R.D., C.Y., D.X.); Department of Biological Sciences, Brock University, St. Catharines, Ontario, Canada L2S 3A1 (X.K, C.D.); and Molecular Plant Physiology, Department of Biology, Faculty of Sciences, Utrecht University, CH–3584 Utrecht, The Netherlands (M.P.)

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5.1 ABSTRACT

In the model plant *Arabidopsis (Arabidopsis thaliana)*, endogenous and environmental signals acting on the shoot apical meristem cause acquisition of inflorescence meristem fate. This results in changed patterns of aerial development seen as the transition from making leaves to the production of flowers separated by elongated internodes. Two related BEL1-like homeobox genes, *PENNYWISE (PNY)* and *POUND-FOOLISH (PNF)*, fulfill this transition. Loss of function of these genes impairs stem cell maintenance and blocks internode elongation and flowering. We show here that *pny pnf* apices misexpress lateral organ boundary genes *BLADE-ON-PETIOLE1/2 (BOP1/2)* and *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA6 (KNAT6)* together with *ARABIDOPSIS THALIANA HOMEBOX GENE1 (ATH1)*. Inactivation of genes in this module fully rescues *pny pnf* defects. We further show that BOP1 directly activates *ATH1*, whereas activation of *KNAT6* is indirect. The *pny pnf* restoration correlates with renewed accumulation of transcripts conferring floral meristem identity, including *FD*, *SQUAMOSA PROMOTER-BINDING PROTEIN LIKE* genes, *LEAFY*, and *APETALA1*. To gain insight into how this module blocks flowering, we analyzed the transcriptome of *BOP1*-overexpressing plants. Our data suggest a central role for the *microRNA156-SQUAMOSA PROMOTER BINDING PROTEIN-LIKE-microRNA172* module in integrating stress signals conferred in part by promotion of jasmonic acid biosynthesis. These data reveal a potential mechanism by which repression of lateral organ boundary genes by PNY-PNF is essential for flowering.
5.2 INTRODUCTION

Plant development relies on the activity of the shoot apical meristem (SAM) as a continuous source of founder cells for production of new leaves, shoots, and internodes throughout the life cycle (for review, see Aichinger et al., 2012). A tight balance between the allocation of cells to developing primordia and the perpetuation of pluripotent stem cells in the central zone maintains the SAM at a constant size. In Arabidopsis (Arabidopsis thaliana), the vegetative SAM produces leaves in a spiral phyllotaxy with dormant axillary meristems. In conjunction, internode elongation is repressed, resulting in a basal rosette. The transition to flowering is governed by internal and external signals that converge at the SAM to promote acquisition of inflorescence meristem (IM) fate (for review, see Amasino and Michaels, 2010; Sríkanth and Schmid, 2011; Andrés and Coupland, 2012). This process, known as floral evocation, results in new patterns of growth at the shoot apex, including production of flowers, and an increase in stem elongation, called bolting. Lateral organ boundaries are specialized domains of restricted growth that separate meristem and organ compartments and produce axillary meristems (for review, see Aida and Tasaka, 2006; Tian et al., 2014). Early in the transition to flowering, the IM produces cauline leaves and axillary meristems that develop as secondary inflorescences. After several nodes, the IM ceases production of leaves, and axillary meristems develop as flowers.

Floral repressors in the SAM block meristem competence to flowering during vegetative stages of development. Major pathways for promotion of flowering work in two ways: by down-regulation of floral repressors in the meristem and by production of factors that promote IM and floral meristem identity (Bernier, 1988; Yant et al., 2010;
Srikanth and Schmid, 2011). The switch to flowering is governed by internal signals, including age, Suc content, and GA, in conjunction with external cues based on photoperiod, vernalization, ambient temperature, and responsiveness to light or stress stimuli (for review, see Srikanth and Schmid, 2011; Wang, 2014). Inputs from these different pathways converge to regulate a number of floral integrator genes, including FLOWERING LOCUS T (FT), which is a central component of the photoperiod response (Srikanth and Schmid, 2011; Andrés and Coupland, 2012). FT encodes a small phosphatidylethanolamine-binding protein that is synthesized in leaves and travels through phloem to the SAM (for review, see Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Andrés and Coupland, 2012), where it interacts with the basic region/leucine zipper motif (bZIP) transcription factor FD to activate genes conferring inflorescence identity, including SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)/AGAMOUS-LIKE20 (AGL20), AGL24, and FRUITFULL (FUL; Abe et al., 2005; Teper-Bamnolker and Samach, 2005; Wigge et al., 2005). These factors in turn promote the expression of floral meristem identity genes LEAFY (LFY), APETALA1 (AP1), and CAULIFLOWER (CAL), which confer floral fate (Bowman et al., 1993). In parallel, age-regulated down-regulation of microRNA156 (miR156) stabilizes mRNA encoding SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL3), SPL4, and SPL5 transcription factors, which function with FT-FD to specify flower development by directly activating AP1, LFY, and FUL expression (Yamaguchi et al., 2009; Jung et al., 2012; Wang, 2014). The plant hormone GA is a positive regulator of flowering with function that is more pronounced under short days (SDs) when other regulatory pathways are inactive. Under SDs, GAs activate the transcription of SOC1 and
LFY in the shoot apex. Under long days (LDs), GA is not required for activation of SOC1 but is important for activation of other transcripts at the shoot apex. Its targets include SPL genes, which are also directly activated by SOC1 and FD (Galvão et al., 2012; Porri et al., 2012). How these various pathways are integrated with stress signals is an area of active study (Yang et al., 2012; Heinrich et al., 2013; Hou et al., 2013; Diallo et al., 2014; Stief et al., 2014).

Members of the THREE-AMINO-ACID-LOOPEXTENSION (TALE) class of homeodomain transcription factors constitute major regulators of meristematic activity. This family includes KNOTTED1-like (KNOX) and BEL1-like (BELL) or BEL1-LIKE HOMEODOMAIN (BLH) members, which function as heterodimers (for review, see Hamant and Pautot, 2010; Hay and Tsiantis, 2010). SHOOT MERISTEMLESS (STM), which is the founding member of the KNOX family in Arabidopsis, is required for SAM initiation and maintenance (Clark et al., 1996; Endrizzi et al., 1996; Long et al., 1996). Other TALE members, such as BREVIPEDICELLUS (BP)/ KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1 (KNAT1), KNAT6, PENNYWISE (PNY; also known as BELLRINGER, REPLUMLESS, VAAMANA, or LARSON), POUND-FOOLISH (PNF), and ARABIDOPSIS THALIANA HOMEBOX GENE1 (ATH1) are expressed in the SAM and contribute redundantly with STM in meristem initiation and maintenance (Byrne et al., 2000; BellesBoix et al., 2006; Rutjens et al., 2009).

PNY contributes to meristem maintenance and flowering with its closest relative, PNF (Smith et al., 2004). During vegetative development, the SAM in pny pnf mutants frequently terminates with development resuming from leaf-derived axillary meristems, a phenotype linked to reduced expression of STM (Smith et al., 2004; Ung et al., 2011; Ung.
and Smith, 2011). The *pny pnf* double mutant is also nonflowering. The *pny pnf* meristem changes shape in response to floral inductive signals, and inflorescence identity genes *SOC1* and *FUL* are up-regulated; however, *FT* levels are reduced, and floral meristem identity genes *LFY*, *AP1*, and *CAL* are not expressed (Smith et al., 2004; Kanrar et al., 2008). The basis of this phenotype is only partly understood. Ectopic expression of *LFY* in *pny pnf* mutants partially rescues flowering at axillary meristems, whereas ectopic expression of *FT* fails to rescue flowering and partially restores internode elongation at length, suggesting that FT requires PNY-PNF to initiate flower development (Kanrar et al., 2008). Additional data show that STM functions in association with PNY-PNF to specify flowers by promotion of *LFY* expression (Kanrar et al., 2006, 2008). This has led to the proposal that STM and PNY-PNF function together with flowering time products *FT-FD* and *AGL24-SOC1* to initiate development of reproductive structures, flowers, and internodes (Smith et al., 2011). More recently, PNY-PNF were shown to promote the expression of SPL3, SPL4, and SPL5 transcription factors that direct activation of floral meristem identity genes in parallel with *FT-FD* (Lal et al., 2011). Compatible with this, miR156 is up-regulated in *pny pnf* apices. Ectopic expression of *SPL4* in *pny pnf* restores accumulation of *LFY* and *AP1* transcripts and partially restores flower formation (Lal et al., 2011). However, none of these mechanisms identified to date fully explain the basis of *pny pnf* meristem defects.

In addition to roles in the SAM, these factors have distinct functions in establishing inflorescence architecture. Significant reorganization of *KNOX-BELL* gene expression occurs at the transition to flowering in correlation with new patterns of aerial development (Lincoln et al., 1994; Byrne et al., 2003; Smith and Hake, 2003; Smith et
al., 2004; Proveniers et al., 2007; Gómez-Mena and Sablowski, 2008). PNY and BP maintain proper internode patterning through the regulation of cell wall remodeling proteins (Mele et al., 2003; Etchells et al., 2012). Mutations in bp cause short internodes and downward-pointing flowers, whereas mutations in pny cause irregular elongation of internodes, leading to clusters of flowers on the primary stem with phenotypes enhanced in the double mutant. Studies in Arabidopsis have identified the joint activities of BLADE-ON-PETIOLE (BOP) Broad Complex, Tramtrack, and Bric-a-brac (BTB)-ankyrin coactivators and TALE homeodomain transcription factors as important in maintaining lateral organ boundaries (for review, see Hamant and Pautot, 2010; Hay and Tsiantis, 2010; Khan et al., 2014). BP and PNY restrict expression of lateral organ boundary genes BOP1/2, KNAT2, KNAT6, and ATH1 to boundaries at the base of the floral shoot in controlling growth patterns in the inflorescence (Ragni et al., 2008; Khan et al., 2012a, 2012b; Zhao et al., 2015). These studies revealed that BOP1/2 promote ATH1 and KNAT6 which form a module that opposes BP-PNY activity in regulating inflorescence architecture (Rutjens et al., 2009; Khan et al., 2012a, 2012b, 2014; Li et al., 2012).

Here, we investigated the interaction of BOP1/2 with TALE members in flower formation. Our studies reveal that PNY and PNF repress the lateral organ boundary genes BOP1/2 and transcriptional targets ATH1 and KNAT6 to maintain meristem integrity and flowering. Inactivation of genes in this module fully rescues pny pnf defects in meristem maintenance, internode elongation, and flowering. To gain insight into how this module blocks flowering, we analyzed the transcriptome of BOP1-overexpressing plants. Our data indicate a role for stress signaling by promotion of jasmonic acid (JA) as a potential
mechanism for counteracting flowering, including responsiveness to GA acting in part through the miR156-SPL-miR172 module.

5.3 RESULTS

5.3.1 Inactivation of BOP1/2, KNAT6, or ATH1 Rescues Meristem Maintenance, Internode Elongation, and Flowering Defects in pny pnf

Previously, we showed that misexpression of boundary genes BOP1/2, KNAT6, and ATH1 in bp and pny internodes perturbs inflorescence architecture through localized restriction of growth. Inactivation of genes in this module fully rescues pny defects in internode elongation and phyllotaxy, but inactivation of KNAT2 has no such effect (Ragni et al., 2008; Khan et al., 2012a, 2012b). We anticipated that antagonistic functions of these same genes might cause pny pnf defects. The pnf single mutant has no obvious phenotype. The pny mutant has a functional SAM, but apical dominance is reduced, flowering is delayed, and organs are clustered on the primary stem because of irregular internode elongation. In pny pnf/+ hemizygous plants, these defects are enhanced, and stem-pedicel fusions occur (Smith and Hake, 2003; Supplemental Fig. S1, A–G). In pny pnf double mutants, the SAM terminates after the initiation of three to five leaves in a majority of seedlings (Smith et al., 2004; Rutjens et al., 2009). Lateral meristems in the axil of rosette leaves support the continued production of leaves, but flowering and internode elongation are blocked (Smith et al., 2004; Rutjens et al., 2009; Lal et al., 2011). To determine if BOP1/2, KNAT/6, and ATH1 are required in generating pny pnf defects, we constructed bop1 bop2 pny pnf, ath1 pny pnf, knat2 pny pnf, knat6 pny pnf, and knat6 knat2 pny pnf mutants. We first tested for rescue of pny pnf defects in SAM
maintenance. Previous studies using the ath1-1 allele indicated that SAM arrest in triple mutants with pny pnf is markedly enhanced, likely because of the depletion of BELL-STM functional complexes (Rutjens et al., 2009). Here, we repeated the analysis with ath1-3, which unlike ath1-1 and ath1-4 alleles, produces no full or partial mutant transcript (Supplemental Fig. S2). Although 57.7% of pny pnf plants showed a meristem arrest, no such arrest was observed in ath1-3 pny pnf mutants (“Materials and Methods”; Fig. 1). Meristem function was also rescued by bop1 bop2 and knat6 mutations but not by inactivation of KNAT2 (Fig. 1). These data suggest that PNY-PNF/STM antagonizes the activity of lateral organ boundary genes to maintain stem cell identity. Flower formation, internode elongation, and organ fusion defects were also rescued in bop1 bop2 pny pnf and knat6 pny pnf or ath1-3 pny pnf triple mutants compared with pny pnf and/or pny pnf/+ plants (Fig. 2, A–H; Supplemental Fig. S3). Quantitative phenotypic analyses showed that inflorescence architecture of bop1 bop2 pny pnf, ath1 pny pnf, and knat6 pny pnf mutants was similar to that of wild-type plants (Supplemental Fig. S4). In contrast, knat2 pny pnf mutants remained nonflowering (Fig. 2I).

Overexpression studies further support a role for BOP1/2, ATH1, and KNAT6 in the same genetic pathway. Plants that overexpress BOP1/2 are late flowering with shortened internodes and clustered fruits similar to pny and pny pnf/+ mutants (Supplemental Fig. S1, A–C; Norberg et al., 2005; Ha et al., 2007; Khan et al., 2012b). Plants overexpressing ATH1 and occasionally, KNAT6 have similar defects that mimic the inflorescence architecture of pny and pny pnf/+ mutants (Supplemental Fig. S1, B–I; Proveniers et al., 2007; Gómez-Mena and Sablowski, 2008; Shi et al., 2011). The most severe KNAT6 transgenic lines were strongly inhibited in their development and failed to
flower (Supplemental Fig. S1, J and K). Collectively, these data indicate that PNY-PNF plays no essential function in meristem/boundary maintenance, internode elongation, and flowering beyond repression of BOP1/2 and ATH1/KNAT6.

5.3.2 BOP1/2, ATH1, and KNAT2/6 Expression Domains Are Expanded in pny pnf Apices

Inflorescence defects in pny mutants correlate with an expanded pattern of expression for BOP1/2, ATH1, and KNAT2/6 in internodes (Ragni et al., 2008; Khan et al., 2012a, 2012b). We therefore examined the expression patterns of these genes in pny pnf apices. In wild-type apices, BOP2 transcripts accumulate in the adaxial domain of floral meristems until late stage 2, when expression shifts to the boundary with the cryptic bract. Expression is found in the boundary domains of older flowers (Fig. 3A; Xu et al., 2010). ATH1 transcripts are expressed in incipient floral primordia and the dome of stage 2 floral primordia in a pattern similar to KNAT2. KNAT6 transcripts are localized to boundary domains flanking the IM and in flowers also overlapping with KNAT2 (Fig. 3, B–D). In pny pnf apices, the domain of expression for all of these genes expands into the central and rib zones of the meristem (Fig. 3, E–H). This was also observed for BOP1 using a BOP1-GUS line (Supplemental Fig. S5). Misexpression of these genes likely begins during the vegetative stage based on analysis of BOP2:GUS lines (data not shown), consistent with SAM structural defects (Ung et al., 2011). Little or no misexpression was observed in pny or pnf control apices (Supplemental Fig. S6). These data confirm that pny pnf defects are caused by misexpression of BOP1/2, ATH1, and KNAT6 in the meristem. We next examined regulatory interactions between these genes in the pathway.
5.3.3 *ATH1* Is a Direct Target of BOP1

BOP1/2 was previously shown to promote the expression of *ATH1* and *KNAT6* and require these activities to exert changes in inflorescence (Khan et al., 2012a, 2012b). To test if *ATH1* and/or *KNAT6* are immediate transcriptional targets of BOP1/2, we used a transgenic line expressing a translational fusion of BOP1 to the steroid-binding domain of the rat glucocorticoid receptor (GR; Lloyd et al., 1994). This dexamethasone (DEX)-inducible system was used previously to show that BOP1 directly activates the transcription of *ASYMMETRIC LEAVES2* in leaves (Jun et al., 2010). Function of the BOP1-GR fusion protein was confirmed by expressing it under the control of a *BOP1* native promoter and observing efficient complementation of *bop1 bop2* leaf and abscission defects upon addition of DEX (Supplemental Fig. S7). Direct regulation of *ATH1* and/or *KNAT6* was tested using the BOP1-GR fusion protein expressed in wild-type plants under the control of a double 35S promoter. *D35S:BOP1-GR* plants treated with DEX for 4 weeks had shortened internodes and clustered fruits similar to *bop1-6D* mutants, which constitutively overexpress *BOP1* (Fig. 4, A–D; Norberg et al., 2005). Transcripts for *ATH1* were increased 13.29-fold and transcripts for *KNAT6* were increased 2.59-fold in *bop1-6D* internodes compared with the wild type (Fig. 4E). Similarly, *D35:BOP1-GR* plants treated with DEX for 4 weeks showed a 6-fold up-regulation of *ATH1* transcript (Fig. 4E). After 2 and 4 h of DEX treatment, transcript levels for *ATH1* were at least 2-fold higher, but *KNAT6* transcript levels showed no increase relative to mock-treated control plants (Fig. 4F; 24-h time point not shown). Rapid activation of *ATH1* suggested that its induction by BOP1 may be direct. We tested this by analyzing *ATH1* and *KNAT6* expression in response to DEX induction in the
presence of the protein synthesis inhibitor cycloheximide (CHX). After 2 and 4 h of combined treatment with DEX and CHX, ATH1 transcripts were increased 5 to 7.5-fold relative to CHX-treated control plants. KNAT6 transcripts were increased up to 2-fold after combined DEX and CHX treatment but not after DEX alone. Presumably, this is an indirect effect of BOP1 dependent on repression of protein synthesis. These data are consistent with ATH1 being a direct target of BOP1 and KNAT6 being an indirect target.

To examine tissue specificity of this interaction, 3.3 and 2-kb ATH1p:GUS reporter genes expressed in D35S:BOP1-GR ("Materials and Methods") were monitored for induction by DEX. Consistent with previous reports (Proveniers et al., 2007; Gómez-Mena and Sablowski, 2008), these reporters were expressed in shoot apices, leaves, and floral organ abscission zones and weakly expressed in the stem. After 4 h of DEX treatment, GUS activity was enhanced relative to mocktreated controls for both promoter lines in all tissues (Fig. 5, A–H). These data confirm that the ATH1 promoter is responsive to BOP1 induction.

BTB-ankyrin proteins, including BOP1/2, have no DNA-binding domain and interact with TGA bZIP binding factors for recruitment to DNA (Després et al., 2000; Hepworth et al., 2005; Xu et al., 2010; Khan et al., 2014). Direct association of BOP1 with the ATH1 promoter was tested by chromatin immunoprecipitation (ChIP) using an anti-GR antibody followed by quantitative reverse transcription (qRT)-PCR. Leaf material was collected from BOP1p:BOP1-GR bop1 bop2 flowering plants. Assays were performed using eight sets of primers spanning 2,178 bp of genomic sequence upstream of the ATH1 transcription start site based on regions enriched in TGA bZIP binding sites (Fig. 5I; see "Materials and Methods"). Motifs that match or closely match consensus
binding sites for TGA factors are also found in the intragenic and 39 untranslated regions of the ATH1 genomic sequence (data not shown). Quantitative analysis by qRT-PCR revealed at least one position in the ATH1 promoter (site IV) showing a reproducible 1.77-fold enrichment of BOP1 protein in DEX-treated plants (Fig. 5J). ChIP assays performed using the mock control showed no significant enrichment at this position or the control UBIQUITIN5 (UBQ5) genomic region. Site IV (nucleotides 22,686 to 22,577) is located approximately 1,515 bp upstream of the ATH1 transcription start site and found within the 3.3-kb ATH1p:GUS construct that is responsive to BOP1 induction in leaves and inflorescences (Fig. 5). Site VII (nucleotides 21,529 to 21,416) was identified as a second potential binding site. Taken together, these data support that BOP1 directly associates with the ATH1 promoter in vivo to regulate its transcription.

5.3.4 Restored Accumulation of Flowering Transcripts in pny pnf Apices after Rescue by Inactivation of BOP1/2, KNAT6, and ATH1

Nonflowering pny pnf apices accumulate SOC1 and FUL transcripts markers of inflorescence identity but fail to accumulate FT or LFY, API, and CAL markers of floral fate (Smith et al., 2004; Kanrar et al., 2008). Accumulation of SPL3, SPL4, and SPL5 transcripts is also diminished in pny pnf apices (Lal et al., 2011). Flowering time of wild-type plants was compared with those of bop1 bop2 pny pnf, knat6 pny pnf, and ath1 pny pnf mutants to further quantify rescue. Figure 6A shows that flowering time for knat6 pny pnf mutants and wild-type control plants was similar. Flowering time of bop1 bop1 pny pnf mutants was slightly delayed (+3.6 d) and flowering time of ath1 pny pnf mutants was slightly earlier (26.9 d) than the wild type, consistent with parental controls (Fig. 6A; Xu et al., 2010). To test if inactivation of BOP1/2, ATH1, and KNAT6 correlates with
restored expression of meristem identity genes in *pny pnf* apices, we measured relative transcript abundance in the wild type and mutants; 25-d-old plants grown under SDs were transferred to LDs to induce flowering. Apices were harvested 12 d later. The floral transition was complete for all genotypes at this time point. Figure 6B confirms that *SOC1* and *FUL* transcripts are relatively unchanged in the wild type compared with mutants. Figure 6B also shows that low to undetectable levels of *FD, LFY, AP1*, and *CAL* transcripts in *pny pnf* apices resumed expression in triple and quadruple mutants, except for *CAL*, which remained low in *bop1 bop2 pny pnf* apices. Transcripts for *FUL, LFY, AP1*, and *CAL* were elevated in *ath1 pny pnf* apices, consistent with earlier flowering. Figure 6C shows that patterns of *miR156* and *SPL* transcript accumulation in triple and quadruple mutants are likewise restored to resemble the wild type. Collectively, these data show that PNY-PNF is dispensable for flowering when BOP1/2, ATH1, and KNAT6 activities are eliminated.

### 5.3.5 *BOP1* Overexpression Mimics *pny pnf* Defects in *SPL* Transcript Accumulation and Responsiveness to GA

Given that *pny pnf* mutants misexpress *BOP1/2*, we used transcript profiling to test if dwarfism and late flowering exhibited by the gain-of-function *bop1-6D* mutant impact similar pathways. We first monitored the accumulation of *miR156* and *SPL* transcripts in *bop1-6D* internodes for comparison with *pny pnf* using qRT-PCR (Fig. 7A). These data show that *miR156* transcripts in *bop1-6D* are 1.4-fold up-regulated relative to the wild type. In addition, *SPL* transcripts in *bop1-6D* were significantly down-regulated, with the exception of *SPL5*. These data suggest that *bop1-6D* partially mimics *pny pnf* (compare Fig. 6C with Fig. 7A).
To further explore similarities and differences between these two mutants, we examined transcripts involved in the regulation of GA, which is a positive regulator of internode elongation and flowering (Mutasa-Göttgens and Hedden, 2009; Porri et al., 2012). The expression levels of genes required for GA biosynthesis and catabolism and DELLA repressors of GA signaling were monitored by qRT-PCR in pny pnf apices and bop1-6D apices and internodes and revealed similar patterns (Fig. 7, B–D). In both genotypes, there was little or no change in ent-kaurene synthase (KS) transcript, but GA20ox1 transcripts were significantly increased (Fig. 7, C and D; Yamaguchi, 2008). In bop1-6D, there was a compensatory decrease in GA3ox1 transcripts functioning later in the biosynthetic pathway (Fig. 7D; Yamaguchi, 2008). In internodes, there was also a compensatory increase in GA2ox7 transcripts required in catabolism (Fig. 7, B and D; Yamaguchi, 2008). All five DELLAs encoding repressors of GA signaling were up-regulated in pny pnf, whereas selective upregulation of REPRESSOR OF GA1-3 LIKE3 (RGL3) was observed in bop1-6D (Fig. 7, C and D). These data indicate that GA homeostasis is disrupted in both mutants. Nevertheless, deficiency alone does not account for phenotypic defects. Spray treatments with GA3 failed to rescue flowering in pny and did not enhance internode elongation in bop1-6D, although this mutant flowered 4 d earlier than mock-treated control plants (Fig. 7, E and F; Smith et al., 2004). In conclusion, SPL transcript accumulation and responsiveness to GA are blocked in both mutants. We, therefore, used microarray analysis of bop1-6D internodes to identify additional factors that might antagonize flowering and internode elongation in these mutants.
5.3.6 Overexpression of BOP1 Activates Stress Pathways and Promotes Accumulation of JA as a Mechanism for Repression of Growth and Flowering

The transcriptomes of bop1-6D versus wild-type internodes were assessed by microarray (“Materials and Methods”). Gene Ontology (GO) analysis of differentially regulated genes revealed significant enrichment of terms associated with response to biotic and abiotic stress stimuli (Supplemental Table S1). Response to JA stimulus (GO:009753) was at the top of the list, but other hormone pathways associated with stress showed similar enrichment. In descending order, these were response to salicylic acid stimulus (GO:0009751), response to ethylene stimulus (GO:009723), and response to abscisic acid stimulus (GO:0099737). These data suggest that bop1-6D plants have heightened expression of stress-related genes. Trade-offs between plant defense and plant growth are well established in the recent literature (Navarro et al., 2008; Wild et al., 2012; Yang et al., 2012; Wild and Achard, 2013), and therefore, we further explored this mechanism. We specifically examined floral repressors in the microarray using a candidate gene approach (Fig. 8A). This analysis revealed up-regulation of DELLA, FLOWERING LOCUS C (FLC)-LIKE (FLC-like), and AP2like members. However, the highest fold changes were observed among AP2/ETHYLENE RESPONSE FACTOR (ERF)-like factors that repress growth and flowering under stress conditions (Magome et al., 2004, 2008; Kang et al., 2011; for review, see Licausi et al., 2013). To validate these findings, selected transcripts were quantified by qRT-PCR using independently isolated tissue samples. Floral repressor transcript profiles of bop1-6D and pny pnf apices genotypes showed strong agreement (Fig. 8B). Consistent with the microarray, no significant change was observed for FLC, but transcripts encoding AP2-like repressors
TARGET OF EAT2 (TOE2; 1.6 to 4-fold) and SCHLAFMUTZE (SMZ; 8.5 to 21-fold) were highly up-regulated compared with the wild type. The highest fold changes (6.2 to 454-fold) were observed for stress-induced AP2/ERF floral repressor transcripts, including **DWARF AND DELAYED FLOWERING1 (DDF1)** and **DDF2**, which encode proteins that inhibit growth by reducing bioactive GA content (Magome et al., 2004, 2008; Kang et al., 2011; for review, see Licausi et al., 2013).

Inspection of the microarray also showed an increase in expression of biosynthetic enzymes for JA (Fig. 9, A and B). Validation of these data by qRT-PCR confirmed significant up-regulation of transcripts involved in JA biosynthesis in **bop1-6D** and **pny pnf** tissues (Fig. 9C). To determine if these increases reflect changes in hormone accumulation in plants, JA levels were quantified in internodes and buds from **bop1-6D** and **pny pnf** apices (“Materials and Methods”). BOP1-overexpressing plants showed 2.5-fold higher levels of JA relative to wild-type plants (Fig. 9D). Conversely, hormone levels were decreased in **bop1 bop2** compared with wild-type control plants. **pny pnf** apices showed 1.5-fold higher levels of JA relative to wild-type control apices at the same stage of development (Fig. 9D). These data suggest that BOP1/2 promotes JA production.

To further examine JA effects on reproductive plant development, methyl jasmonate (MeJA) was applied to wild-type and **pny** plants grown under LDs (Fig. 10). Plants of both genotypes treated with MeJA developed a compact rosette with small dark green leaves, similar to those of **bop1-6D** mutants (Fig. 10, A–C). Wild-type plants treated with MeJA showed partial loss of apical dominance similar to **pny** mutants (Fig. 10, D–G). Plants in both treatment populations were late flowering with short internodes relative to mock-treated control plants (Fig. 10, D–G) and similar to **pny pnf/−** mutants.
(Supplemental Fig. S1, A–G). Organ fusions or clusters were not observed. In both wild-type and pny populations, a small subset of plants developed a disordered rosette phenotype similar to pny pnf mutants and were nonflowering after 10 weeks (data not shown). No such defects were observed in mock-treated control plants. Thus, treatment of wild-type plants with exogenous MeJA mimics the phenotype of bop1-6D and pny or pny pnf/+ plants.

In parallel, we tested if reducing JA content rescues internode elongation or flowering in pny pnf and/or bop1-6D mutants by crossing them to the allene oxide synthase (aos) mutant, which is defective JA synthesis (Park et al., 2002; Figs. 7B and 10). Triple mutants with pny pnf remained nonflowering, even with addition of exogenous GA3 (Fig. 10H; data not shown). However, quantitative analysis of bop1-6D aos double mutants revealed a small but significant (P # 0.0001) increase in flowering time (+1.8 d) and plant height (+1.5 cm) compared with bop1-6D siblings in a segregating population (Fig. 10, I and J). These data provide evidence that modulation of growth by JA is a potential factor in conditioning bop1-6D and pny pnf phenotypic defects.

5.4 DISCUSSION

Floral evocation is dependent on SAM restructuring to form an IM (Bernier, 1988). The TALE homeodomain PNY and PNF transcription factors are essential for this process by permitting responsiveness to floral inductive signals (Smith et al., 2004; Kanrar et al., 2008; Lal et al., 2011; Smith et al., 2011; Ung et al., 2011; Ung and Smith, 2011). In pny pnf mutants, meristems support the production of leaves, but internode
elongation and flower initiation are blocked.

In this article, we characterized the interaction of PNY and PNF with lateral organ boundary factor BOP1/2 and a pair of downstream effectors: the KNOX-BELL homeodomain factors KNAT6 and ATH1. We show that misexpression of these genes in pny pnf apices blocks floral evocation (Fig. 11). Inactivation of BOP1/2 and ATH1 or KNAT6 fully restores pny pnf defects in meristem and boundary maintenance and stem elongation and restores expression of floral meristem identity genes to allow flowering. Remarkably, other factors compensate for the loss of these genes in maintaining the SAM and responsiveness to floral inductive signals. Thus, PNY and PNF allow flowering by excluding boundary genes from the meristem. Similar antagonistic interactions for PNY or BP with members of the BOP1/2-ATH1/KNAT6 module function in various other developmental contexts, including abscission, fruit patterning, and inflorescence architecture (Ragni et al., 2008; Shi et al., 2011; Khan et al., 2012a, 2012b; Li et al., 2012).

We further investigated the organization of this module and its transcriptional targets. Our data show that BOP1 is a direct regulator of ATH1, whereas promotion of KNAT6 is probably indirect. Indeed, DEX and CHX treatment of 35S:ATH1-GR plants produces rapid induction of KNAT6 transcript, and reporter gene expression is missing at boundaries in ath1-3 but not bop1 bop2 mutants, suggesting a direct requirement for ATH1 (data not shown). BOP1/2 coactivators are recruited to DNA through interactions with TGA bZIP transcription factors (Hepworth et al., 2005; Xu et al., 2010). These TGA factors remain unknown in the context of flowering, but several candidates are being investigated (Fig. 11). Transcript profiling was used to probe how this module blocks
flowering. Comparison of the gain-of-function \textit{bop1-6D} mutant and \textit{pny pnf} showed similar transcriptional defects in core pathways controlling flowering. Our data are consistent with the model that BOP1/2-ATH1/KNAT6 boundary genes activate stress pathways that promote JA biosynthesis, which directly or indirectly interferes with signals integrated by the \textit{miR156-SPL-miR172} module to antagonize IM function (Fig. 11). Details of this model are discussed below.

\textbf{5.4.1 The \textit{miR156-SPL-miR172} Module as a Hub for Integration of Flowering Signals}

The \textit{miR156-SPL-miR172} module is a core pathway for integration of flowering signals, including age, sugar, GA, and stress (Huijser and Schmid, 2011; Cho et al., 2012; Proveniers, 2013; Cui et al., 2014; Stief et al., 2014; Wang, 2014). In brief, \textit{miR156} levels decline with age, leading to a concomitant increase in abundance of \textit{SPL} transcripts with products that act on distinct targets in leaves and the shoot apex to promote flowering (Wu and Poethig, 2006; Wang et al., 2009; Wu et al., 2009). \textit{SPL3} and \textit{SPL9} members in the SAM directly promote the activation of floral meristem identity genes (Wu et al., 2009; Yamaguchi et al., 2009). \textit{SPL9}-like members have additional functions in leaves, where they activate the transcription of \textit{miR172b}, which lowers the abundance AP2-like floral repressor transcripts and allows accumulation of \textit{FT} mRNA (Zhu and Helliwell, 2011; Matsoukas et al., 2012; Wang, 2014).

Significant reduction of \textit{miR156}-regulated \textit{SPL} transcripts was observed in \textit{pny pnf} and \textit{bop1-6D} mutants. This reduction is likely driven by multiple factors, including lower levels of FD, which recruits FT to the promoter of \textit{SPLs} for activation (Jung et al., 2012; Andrés et al., 2015), and higher steady-state levels of \textit{miR156} (Lal et al., 2011). An
increase in \textit{miR156} was less marked in \textit{bop1-6D}, suggesting that the reduction in \textit{SPL} transcript is mediated by \textit{miR156} and other regulators. These data are consistent with previous work showing that \textit{SPL3/4/5} transcripts are reduced in \textit{pny pnf} apices and partly account for nonflowering (Lal et al., 2011). Transgenic \textit{pny pnf} plants expressing an \textit{miR156}-resistant form of \textit{SPL4} were restored for \textit{LFY} and \textit{AP1} expression but only partly restored for flowering, suggesting that multiple \textit{SPL} factors are involved (Lal et al., 2011).

Concomitantly, transcripts encoding \textit{miR172}-regulated AP2-like repressors of flowering and internode elongation were elevated in \textit{bop1-6D} and \textit{pny pnf} mutants. This group of repressors includes AP2, SMZ, TOE1, TOE2, and OE3 with overlapping functions (Aukerman and Sakai, 2003; Jung et al., 2007; Mathieu et al., 2009; Yant et al., 2010). SMZ and presumably, other members of this family delay flowering through the direct repression of \textit{FT} and promotion of \textit{miR156} (Mathieu et al., 2009; Yant et al., 2010). Of these, \textit{TOE2} and \textit{SMZ} show consistent upregulation in the transcriptome of \textit{bop1-6D} and \textit{pny pnf} apices. Thus, overexpression of AP2-like members in \textit{bop1-6D} may be a route to restricting internode elongation and flowering.

\textbf{5.4.2 Integration with Signals for Stress and Carbohydrate Metabolism}

Stress and sugar signals are also integrated through the \textit{miR156-SPL-miR172} module to control flowering (for review, see Wang, 2014). Recent studies address the mechanism. One study shows that \textit{miR156-SPL3} delays flowering under cool ambient temperatures by regulation of \textit{FT} (Kim et al., 2012). Similarly, plants overexpressing \textit{miR156} are late flowering with increased tolerance to stress linked to down-regulation of \textit{SPL9} (Cui et al., 2014). Stief et al. (2014) further showed that heat stress induces \textit{miR156}
isoforms linked to downregulation of SPL9-like transcripts (SPL2, SPL9, and SPL11) and delayed flowering. Induction of miR156h in this cascade is predicted to target the pectin methylesterase inhibitor At5g38610, which may affect bolting (Stief et al., 2014). PNY controls inflorescence patterning by regulating cell wall modification enzymes, including pectin methylesterases, which loosen cell walls in the stem to promote internode elongation and in the SAM to facilitate organ initiation (Etchells et al., 2012; Peaucelle et al., 2011). At5g38610 and related genes are up-regulated in the transcriptome of bop1-6D internodes, whereas PNY-regulated PECTIN METHYLESTERASE5 is down-regulated, consistent with dwarf stature (data not shown; Peaucelle et al., 2011).

The miR156-SPL-miR172 module is also a sensor for nutrients. A developmental decline in miR156 is partially mediated by sugars produced by photosynthesis that accumulate with age (Proveniers, 2013; Yang et al., 2013; Yu et al., 2013). Global transcript changes in bop1-6D mutants are characterized in large part by alterations in stress signaling and carbohydrate metabolism (Supplemental Table S1). GO enrichment analysis of the bop1-6D transcriptome identifies significant down-regulation of cellular carbohydrate metabolism, metabolic processes, and nitrogen metabolism, which potentially act to restrict Suc availability at the shoot apex (Supplemental Table S1). Parts of these changes were confirmed in pny pnf mutants, suggesting that resources are allocated toward defense in detriment to flowering.

5.4.3 Integration with GA Pathways

Our study also identifies GA pathway changes in bop1-6D and pny pnf mutants detrimental to flowering. In wild-type plants, bioactive GA content increases 100-fold at the transition (Eriksson et al., 2006), facilitating internode elongation and flowering by
lowering the abundance of DELLA repressors (Mutasa-Göttgens and Hedden, 2009; Galvão et al., 2012; Porri et al., 2012; Yu et al., 2012). GA signals are partly integrated through the \textit{miR156-SPL-miR172} module based on studies showing that GA/DELLA regulates \textit{SPL3/4/6/9} transcription at the shoot apex independent of SOC1 (Galvão et al., 2012; Porri et al., 2012). Physical interaction of REPRESSOR OF GA1-3 DELLA with SPL9 interferes with activation of MADS-box flowering genes at the shoot apex and activation of \textit{miR172b} in leaves, thereby maintaining AP2 and AP2-like repression of stem elongation and flowering (for review, see Wang, 2014). Other nodes of integration with the \textit{miR156-SPL-miR172} module are likely given so that GA treatment does not markedly accelerate flowering in an \textit{miR156} overexpression line (Yu et al., 2012). Transcriptional profiling in \textit{bop1-6D} and \textit{pny pnf} plants indicates complex changes affecting biosynthesis, catabolism, and/or signaling. Exogenous GA fails to restore flowering in \textit{pny pnf} apices or internode elongation in \textit{bop1-6D}, similar to transgenic plants overexpressing ATH1 (Smith et al., 2004; Gómez-Mena and Sablowski, 2008; this study) and consistent with blockage at multiple steps. Four of five DELLA transcripts are significantly up-regulated in \textit{pny pnf} apices, whereas \textit{RGL3} is selectively up-regulated in \textit{bop1-6D}. Transcript accumulation and steady-state level of protein show strong correlation in previous studies (Wild et al., 2012). Transgenic plants overexpressing DELLAs or DELLA proteins resistant to degradation are dwarf and late flowering, similar to \textit{bop1-6D} plants (Dill et al., 2004; Hamama et al., 2012). RGL3, in particular, mediates cross talk between GA and JA pathways (Hou et al., 2013; Wild and Achard, 2013). JA selectively upregulates RGL3, which binds to jasmonate ZIM-domain repressors of JA signaling to boost the immune response at the expense of growth (Wild
et al., 2012; Wild and Achard, 2013).

5.4.4 JA Antagonism of Growth and Flowering

Our data raise the interesting possibility that JA antagonism of GA conditions bop1-6D and pny pnf phenotypic defects. GO analysis of differentially regulated genes in the bop1-6D transcriptome revealed significant enrichment of terms related to stress stimuli, including response to JA stimulus and to a lesser extent, responses to salicylic acid, ethylene, and abscisic acid stimuli, leading to the model that BOP1 overexpression reprioritizes the plant for defense at the expense of growth. Higher levels of JA biosynthetic gene transcripts and hormone are found in bop1-6D and pny pnf apices relative to wild-type control plants. These data support the findings by Canet et al. (2012), which identified BOP1/2 as essential for MeJA induced in priming for resistance to Pseudomonas syringae pv tomato DC3000. Plants exposed to high levels of jasmonate are stunted in growth of roots, leaves, and stems (Ellis et al., 2002; Cipollini, 2005; Bonaventure et al., 2007; Hyun et al., 2008; Zhang and Turner, 2008; Heinrich et al., 2013). Arabidopsis plants treated with jasmonate are also late flowering with short internodes and loss of apical dominance, giving an appearance similar to bop1-6D or pny pnf/+ mutants. Inhibitory effects of MeJA on flowering are also reported in Pharbitis nil (Maciejewska and Kopceiwicz, 2002; Maciejewska et al., 2004), Chenopodium rubrum (Albrechtová and Ullmann, 1994), and einkorn wheat (Triticum monococcum; Diallo et al., 2014). JA antagonism of growth or flowering has been linked to repression of GA biosynthesis (Magome et al., 2004; Heinrich et al., 2013), stabilization of DELLAs (Yang et al., 2012), and/or induction of AP2/ERF factors (Magome et al., 2008; Sun et al., 2008; Kang et al., 2011; Licausi et al., 2013). These data are consistent with JA contributing to
bop1-6D and pny pnf developmental defects. Although inactivation of jasmonate biosynthesis by mutation of AOS fails to rescue flowering in pny pnf mutants, a small but significant increase in plant height and flowering time in bop1-6D supports this model.

Our data suggest that resources in pny pnf are reallocated toward defense at the expense of flowering and provide evidence for JA as a factor in modulating growth and meristem activity at boundaries.
5.5 METHODS

5.5.1 Plant Material and Growth Conditions

In the laboratory of S.R.H., *Arabidopsis (Arabidopsis thaliana)* plants were grown on soil or in vitro on minimal media (Haughn and Somerville, 1986) in growth chambers at 21°C under continuous light (24 h of light; intensity of 100 µmol m\(^{-2}\) s\(^{-1}\)), LD (16 h of light), or SD (8 h of light) conditions. In the laboratory of V.P., plants were grown in LD (16 h of light; 150 µmol m\(^{-2}\) s\(^{-1}\)) or SD (10 h of light; 1 h at 80 µmol m\(^{-2}\) s\(^{-1}\); 8 h at 130 µmol m\(^{-2}\) s\(^{-1}\), and 1 h at 80 µmol m\(^{-2}\) s\(^{-1}\)) conditions. The wild type was the Columbia (Col-0) ecotype of *Arabidopsis*. Mutant lines were obtained from the *Arabidopsis* Biological Resource Center (https://abrc.osu.edu/) or the Nottingham *Arabidopsis* Stock Centre (http://arabidopsis.info/). The *pny-40126* (SALK_40126), *pnf-96116* (SALK_96116), *bop1-3* (SALK_012994), *bop2-1* (SALK_075879), *knat6-1* (SALK_047931), *knat6-2* (SALK_054482), *knat2-5* (SALK_099837), *ath1-1* (GABIKAT_114A12), and *ath1-3* (SALK_113353) mutants have been described previously (Smith and Hake, 2003; Smith et al., 2004; Hepworth et al., 2005; Belles-Boix et al., 2006; Proveniers et al., 2007; Gómez-Mena and Sablowski, 2008). The *ath1-4* mutant was a gift from Lin Xu (Li et al., 2012). 35S:BOP2 and *bop1-6D* overexpression lines were described previously (Norberg et al., 2005). The *BOP1:GUS* and *BOP2:GUS* reporter lines were described previously (McKim et al., 2008; Xu et al., 2010). The 35S:KNAT6 overexpression line was also described previously (Shi et al., 2011).

5.5.2 Plant Genetics

Primers and strategies used for genotyping *bop1-3*, *bop2-1*, *knat6-2* (Khan et al., 2012b), *pny-40126* (Smith and Hake, 2003), *pnf-96116*, *pnf-33879* (Smith et al., 2004), *knat6-1*, *knat2-5* (Ragni et al., 2008), *ath1-1* (Proveniers et al., 2007), and *ath1-3* (Gómez-Mena and Sablowski, 2008) have been previously described. For genotyping *ath1-4*, primers *ath1-4dCAPS-F* and *ath1-4dCAPS-R* were used to amplify a 198-bp product from genomic DNA. Only the *ath1-4* product is cleaved by SspI to yield a 173-bp fragment. All mutant combinations were generated by crossing and confirmed by PCR genotyping. Primers are listed in Supplemental Table S2.

5.5.3 Phenotypic Analyses

For quantitative analysis of meristem arrest, seedlings were germinated on agar plates under SDs, transferred to soil on day 10, and scored for meristem arrest on day 25. Progenies from a selfed *pny pnf/+* plant (n = 624) and a selfed *knat2 pny pnf/+* plant (n = 146) were analyzed in parallel with wild-type plants and *bop1 bop2 pny pnf*, *ath1 pny pnf*, and *knat6 pny pnf* mutants (n = 144). Quantitative analyses of inflorescence phenotypes were performed with 8-week-old plants grown under LDs. Average height, internode length, and rosette paraclade number were determined for 10 plants per genotype as previously described (Ragni et al., 2008). Flowering time was scored for at least 24 plants per genotype by monitoring the date of apex emergence, because *bop1 bop2* mutants...
initiate leaves at a reduced rate (Norberg et al., 2005). Seeds were germinated directly on soil under LDs. All phenotypic analyses were performed at least twice under independent growth conditions with similar results.

5.5.4 In Situ Hybridization and Localization of GUS Activity

Plants for analysis were grown under SDs for 3 weeks followed by 15 d in continuous light before harvesting tissue. We used in situ hybridization to monitor gene expression, because control sequences for expression of KNAT2:GUS and KNAT6:GUS reporters in IMs are missing (Khan et al., 2012b). Tissue fixation, embedding, and sectioning were carried out as described (Nikovics et al., 2006) with minor changes. Hybridization was performed overnight using the following buffer: 50% (v/v) formamide, 10% (w/v) dextran sulfate, 1x Denhardts, 0.3 M NaCl, 10 mM Tris HCl, pH 8, 1 mM EDTA, and 5 mg mL\(^{-1}\) transfer RNA. Primers used to make KNAT6, KNAT2, BOP2, and ATH1 antisense probes are as listed in Supplemental Table S2.

Tissues were analyzed for BOP1:GUS activity as described (Sieburth and Meyerowitz, 1997) with minor changes. Stained tissues were embedded in Paraplast Plus (Sigma) processed using tert-butanol instead of xylenes. Sections (10 µm) were cut from embedded tissue, affixed to glass slides, and dewaxed with tert-butanol before imaging.

5.5.5 Construction of D35S:BOP1-GR, BOP1p-BOP1-GR, D35S: ATH1, and ATH1p:GUS Transgenic Lines

A translational fusion of BOP1 to the steroid-binding domain of the rat glucocorticoid receptor was generated. Treatment with DEX leads to translocation of the GR fusion protein from the cytoplasm to the nucleus as a way of controlling transcription factor activity (Lloyd et al., 1994). The BOP1 coding sequence lacking a stop codon was fused in frame to the GR fragment using overlap extension mutagenesis (Heckman and Pease, 2007). The resulting product was cloned into pCR-BluntII-TOPO (Invitrogen) to create B359. For all cloning steps involving amplification by PCR, iProof was used as the polymerase (BioRad), and cloned inserts were sequenced to ensure fidelity.

To create D35S:BOP1-GR, the BOP1-GR fusion gene present in B359 was amplified by PCR using CDS-BOP1-F and GR-R as the primers. The resulting product was modified to contain dATP overhangs and transferred to the Gatewaycompatible entry vector pCR8/GW/TOPO (Invitrogen). LR clonase (Invitrogen) was used to move the insert to a pSM-3-based destination vector containing a double 35S Cauliflower mosaic virus (CaMV) promoter (D35S) and Nos terminator (pBAR, gift of C. Douglas). Wild-type plants were transformed by floral dipping (Clough and Bent, 1998) using the Agrobacterium spp. strain C58C1 pGV3101 pMP90 (Koncz and Schell, 1986). Hygromycin-resistant primary transformants were selected on agar plates containing 10 µm of DEX. After transfer to soil, plants were sprayed daily with 10 µm of DEX to induce nuclear localization of the BOP1-GR fusion protein. Homozygous progeny from one DEX-induced D35S:BOP1-GR line with a dwarf phenotype (line 9) was used for all subsequent experiments.

The D35S:BOP1-GR transgene failed to complement bop1 bop2 plants, presumably because the 35S CaMV promoter fails to provide the correct range of tissue
expression. To confirm activity of the fusion protein and for use in ChIP experiments, the BOP1-GR fusion gene was expressed under control of the BOP1 native promoter in bop1 bop2 plants. The transgene was created in two steps. The BOP1 promoter present in pBOP1:GUS (McKim et al., 2008) was amplified by PCR using primers 4H-4kb-EcoR1-F1 and 4H-4kb-XmaI-R1 that incorporated restriction sites at their 5' ends. The resulting product was digested with EcoR1 and XmaI and cloned into the corresponding sites of the binary vector pBAR (gift from laboratory of J. Dangl) to create B149. The BOP1-GR fusion gene present in B359 was amplified by PCR using primers XmaI-BOP1-F and BOP1-XmaI-R. The resulting product was digested with XmaI and cloned into the corresponding site of B149 to create pBAR/BOP1prom:BOP1-GR. The transgene was introduced into bop1 bop2 plants by floral dipping. Primary transformants resistant to glufosinateammonium were selected on soil using the herbicide FINALE (Farnam Companies). Three independent lines were used to assess complementation of bop1 bop2 mutant phenotypes. T2 seeds were sown on agar plates containing phosphinothricin with or without 5 µm of DEX. Plants were transferred to soil and sprayed daily with mock or DEX solution until maturity. Complementation of leaf, floral patterning, and floral organ abscission was observed in all DEX-treated lines (Supplemental Fig. S7).

To make the D35S:ATH1 transgene, the ATH1 coding sequence was amplified by PCR from cloned complementary DNA (cDNA) template using ATH1-CDS-F1 and ATH1-CDS-F1 as the primers. The resulting fragment was cloned into the entry vector pCR8/GW/TOPO and transferred into the pSM-3-based destination vector as described above. Wild-type plants were transformed by floral dipping. Transformants were selected on agar plates containing hygromycin. Phenotypes were scored in the T1 generation.

To create ATH1 promoter fusions to a GUS reporter gene, fragments containing 3.3 or 2 kb of sequence upstream of the ATH1 translation start site were amplified by PCR from genomic DNA template (BAC MSD21) and fused to the coding region of the beta-glucuronidase (uidA or GUS) gene. Primers incorporating BamHI and NcoI restriction sites at their 5' ends facilitated directional cloning. Products were cloned into pCR-BluntII-TOPO for propagation. Inserts were released by digestion with BamHI and NcoI and ligated into the corresponding sites of pGCO:GUS (Hepworth et al., 2002). Agrobacterium spp. was cotransformed with pSOUP (Hellens et al., 2000). Wild-type plants were transformed by floral dipping, and glufosinate-ammonium-resistant primary transformants were selected on soil. Cloning primers are listed in Supplemental Table S2.

5.5.6 ChIP Experiments

ChIP was performed as described (Chakravarthy et al., 2003) using an anti-GR antibody (catalog no. 1002; Santa Cruz Biotechnology) and mockor DEX-treated BOP1p:BOP1-GR bop1 bop2 plants grown under LDs. Seeds were germinated on agar plates containing phosphinothricin with or without 10 µm of DEX. After transfer to soil, plants were sprayed daily with mock (0.04% ethanol) or DEX solutions. Leaf tissue was collected from 4-week-old flowering plants for analysis. Quantification of immunoprecipitated DNA by qRT-PCR was performed as previously described (Boyle et al., 2009). Primers were as listed in Supplemental Table S3.
5.5.7 Microarray Experimental Design, Hybridization, and Analysis

Tissue for profiling was harvested from the first expanded internodes of wildtype and \textit{bop1-6D} flowering plants grown under continuous light. RNA was extracted from four biological replicates per genotype using an RNeasy Plant Mini Kit (Qiagen). The mRNA was amplified according to the protocol described in the MessageAmp aRNA Kit (catalog no. 1750; Ambion). To produce incorporated antisense mRNA, aminoallyl-UTP was incorporated into the newly synthesized RNA; 3 mL of aminoallyl-UTP (50 mM) plus 2 mL of UTP (75 mM) instead of 4 mL of UTP were added during the aRNA amplification. Labeling, hybridization, and scanning were performed as described (Xiang et al., 2011). To normalize for bias in dye labeling, two biological replicates were labeled with [59-32P] cytosine-39-P (Cy3), and two were labeled with Cy5. Experiments were carried out using \textit{Arabidopsis} 70-mer oligo microarray slides (http://ag.arizona.edu/microarray). Two-color microarray data were preprocessed with the marray package (version 1.42.0) implemented in R/BioConductor (R Development Core Team; Gentleman et al., 2004; https://www.bioconductor.org) using the background correction method normexp (offset 5 50) and normalize within arrays method loess. Differentially expressed genes were identified by P values, fold changes, and contrasts using linear models for microarrays (Smyth, 2005) and included a dye effect assessment implemented in R/BioConductor.

5.5.8 qRT-PCR

Total RNA was isolated using Trizol Reagent (Invitrogen) from dissected apices of the wild type and mutants. Plants grown under SDs were harvested on day 25 (SD) or transferred to LDs to induce flowering and harvested after 12 d (LD). Dissected apices were \(0.5\) cm tall, with the majority of surrounding leaves \(0.2\) cm removed. Tissues were collected in the subjective afternoon for all samples (after 9–12 h of light in a 16-h cycle). cDNA was generated using 1 mg of RNA as the template under following conditions: step 1: 70°C for 5 min; step 2: 50°C for 60 min; and step 3: 70°C for 15 min. qRT-PCR was carried out as described (Khan et al., 2012b) with the following changes. Reactions in triplicate containing 2 mL of 10-fold diluted cDNA, except for LFY and AP1 reactions, which required 4 mL of diluted cDNA, gene-specific primers (Supplemental Table S3), and POWER SYBR Green PCR Mastermix (Invitrogen) were carried out using a StepOnePlus Thermocycler (Applied Biosystems). \textit{GLYCERALDE-3-PHOSPHATE DEHYDROGENASE} \(C\) was used as a normalization control. Quantification of \textit{miR156} mRNA was performed as described (Porri et al., 2012). Data shown are the average of three biological replicates conducted using separate growth trials and independently isolated RNA samples. Error bars indicate SEM.

For DEX induction experiments, total RNA was prepared from internodes of 4-week-old flowering plants expressing the \textit{D35S:BOP1-GR} transgene. Internodes were harvested from primary and secondary inflorescences of five to six plants starting at the bottom above the first silique and going all of the way up to where internodes were too small to collect. Tissue was excised with a new razor blade on parafilm, frozen in liquid nitrogen, and stored at 280°C until further analysis. Plants were treated continuously with mock (0.12% ethanol), 30µm of DEX, 50µm of CHX, or 30µm of DEX and 50µm of
CHX for 2, 4, or 24 h by inverting inflorescences into containers of solution. For long-term treatments, seedlings were germinated on agar plates containing 10 µm of DEX. After transplanting to soil, plants were sprayed daily with a solution of mock (0.04% ethanol) or DEX for 4 weeks until tissue was harvested for RNA extraction. Values were normalized to EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1 transcript (At3g13920), the mock control for DEX treatments, and the CHX control for DEX and CHX treatments to correct for negative effects of CHX on the transcription of BOP1 target genes (Jun et al., 2010; Nakamichi et al., 2010). Data shown are the average of three biological replicates conducted using independently isolated RNA samples. Error bars indicate SEM.

5.5.9 Hormone Treatments

To analyze the effect of GA on growth, 10-d-old seedlings grown under continuous light were sprayed daily with GA (100 µm of GA₃ and 0.02% Silwett L-77) or a mock (0.02% Silwett L-77) solution until maturity (Hay et al., 2002). To examine the effect of JA on growth, 7-d-old seedlings grown under LDs were sprayed daily with MeJA (100 µm of MeJA and 0.02% Silwett L-77) or a mock (0.02% Silwett L-77) solution until maturity (Canet et al., 2012). MeJA-treated plants were covered with a plastic dome for 1 h after treatments, and solutions were made fresh once a week. Flowering time was determined by scoring the date of apex emergence. At least 24 plants per genotype were monitored.

5.5.10 JA Measurements

For measurement of JA, wild-type, bop1 bop2, and bop1-6D plants were grown for 6 to 7 weeks under LDs. Pools of 30 apices (buds and internodes) were used for each replicate (100 mg of fresh material). Wild-type and pny pnf plants were grown for 4 weeks under SDs. Pools of 30 apices (90 mg of fresh material) were used for each replicate. Three biological replicates were collected for each condition. Tissues were directly harvested in liquid nitrogen. Tissues were ground in liquid nitrogen and lyophilized. For each sample, 10 mg of freeze-dried powder was extracted with 0.8 mL of acetone:water:acetic acid (80:19:1, v/v/v) containing 2 ng of [5-2H] JA (CDN Isotopes CIL Cluzeau; Le Roux et al., 2014). The extract was vigorously shaken for 1 min, sonicated for 1 min at 25 Hz, shaken for 10 min at 4°C in a Thermomixer (Eppendorf), and then centrifuged (8,000g at 4°C for 10 min). The supernatants were collected, and the pellets were reextracted twice with 0.4 mL of the same extraction solution; then, they were vigorously shaken (1 min) and sonicated (1 min; 25 Hz). After the centrifugations, the three supernatants were pooled and dried (final volume of 1.6 mL). Each dry extract was dissolved in 140 mL of acetonitrile:water (50:50, v/v), filtered, and analyzed using a Waters Acquity Ultra Performance Liquid Chromatograph coupled to a Waters Xevo Triple Quadrupole Mass Spectrometer TQS. The compounds were separated on a reverse-phase column (100 mm × 2.1 mm × 3 mm particle size; Uptisphere C18 UP3HDO; Interchim) using a flow rate of 0.4 mL min⁻¹ and a binary gradient: 0.1% (v/v) acetic acid in water and acetonitrile with 0.1% acetic acid. For JA, the following binary gradient (0.1% [v/v] acetic acid in water) was used: 0 min, 98%; 3 min, 70%; 7.5 min, 50%; 8.5 min, 5%; 9.6 min, 0%; 13.2 min, 98%; and 15.7 min, 98%. Mass
spectrometry was conducted in electrospray and multiple reaction monitoring scanning mode in negative ion mode. Relevant instrumental parameters were set as follows: capillary, 1.5 kV (negative mode); source block and desolvation gas temperatures, 130°C and 500°C, respectively. Nitrogen was used to assist the cone and desolvation (150 and 800 L h\(^{-1}\), respectively). Argon was used as the collision gas at a flow of 0.18 mL min\(^{-1}\). The parameters used for multiple reaction monitoring quantification of JA are described in Le Roux et al., 2014. Samples were reconstituted in 140 mL of 50:50 (v/v) acetonitrile:water per 1 mL of injected volume. The JA limit of detection and limit of quantification were extrapolated from calibration curves and samples using the Quantify module of MassLynx software (version 4.1). The amount of JA was expressed as a ratio of peak areas (209 > 62/214 > 62) per dry weight because of impurities contained in the D5 JA standard.

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers At1g70510 (KNAT2), At1g23380 (KNAT6), At5g02030 (PNY), At2g27990 (PNF), At3g57130 (BOP1), At2g41370 (BOP2), and At4g32980 (ATH1).
Figure 1. Inactivation of BOP1/2, ATH1, and KNAT6 rescues pny pnf meristem arrest.

Plants were grown under SDs. Numbers of plants showing a meristem arrest on day 25 are indicated at the upper right. A, Col-0 plant. The SAM produces leaves. B, pny pnf mutant showing a meristem arrest; 90 of 156 (57.7%) of expected pny pnf mutants in a pny pnf/+ segregating population (n = 624) showed SAM arrest (arrow). C, knat2 pny pnf triple mutant (identical to pny pnf mutant); 11 of 36.5 (30.1%) of expected knat2 pny pnf triple mutants in a knat2 pny pnf/+ segregating population (n = 146) showed SAM arrest (arrow). D, ath1 pny pnf triple mutant (no meristem arrest). E, bop1 bop2 pny pnf quadruple mutant (no meristem arrest). F, knat6 pny pnf triple mutant (no meristem arrest). Bars = 5 mm.
Figure 2. Inactivation of BOP1/2, ATH1, and KNAT6 rescues internode and flower formation in pny pnf mutants.

Representative 8-week-old plants are shown. A, Col-0 plant. B, pnf mutant showing a wildtype phenotype. C, pny mutant showing partial loss of apical dominance, short stature, and clusters of siliques. D, pny pnf/+ hemi mutant showing partial loss of apical dominance, short stature, clusters of siliques, and stem/pedicel fusion defects (Supplemental Fig. S1). E, pny pnf double mutant (nonflowering). F, bop1 bop2 pny pnf quadruple mutant (similar to bop1 bop2). Inactivation of BOP1 and BOP2 in pny pnf rescues internode elongation and flowering. G, ath1 pny pnf triple mutant (similar to ath1). Inactivation of ATH1 in pny pnf rescues internode elongation and flowering. H, knat6 pny pnf mutant (similar to the wild type). Inactivation of KNAT6 in pny pnf rescues internode elongation and flowering. I, knat2 pny pnf mutant (identical to pny pnf mutant). Bars = 2 cm.
Figure 3. BOP2, ATH1, KNAT2, and KNAT6 expression in pny pnf apices.

Plants were grown for 3 weeks under SDs and transferred to continuous light to induce flowering. Apices were harvested on day 15. Transcript accumulation was monitored by in situ hybridization using longitudinal sections of Col-0 (A–D) and pny pnf (E–H) apices and gene-specific probes. Numbers in panels indicate the stage of floral development (Smyth et al., 1990). A, Col-0 apex showing BOP2 expression in floral meristems (until stage 2) and the boundary domains of older flowers (late stage 2 and stage 3 are shown). B, Col-0 apex showing ATH1 expression in an incipient floral primordium and the dome of a stage 2 flower. C, Col-0 apex showing KNAT2 transcripts localized to boundary domains flanking the IM and older flowers. Expression is also observed in floral primordia and the dome of stage 2 flowers. D, Col-0 apex showing KNAT6 transcripts localized to boundary domains flanking the IM and in a stage 3 flower. E to H, pny pnf apices showing expanded expression of BOP2 (E), ATH1 (F), KNAT2 (G), and KNAT6 (H) in the central and rib zones of the meristem. Bars = 40 µm.
Figure 4. Activation of *ATH1* and *KNAT6* in the DEX-induced *D35S:BOP1-GR* line.

A, Col-0 plant. B, *bop1-6D* mutant with shortened internodes and clustered siliques. C and D, *D35S:BOP1-GR* plants treated with mock or DEX solutions for 4 weeks. C, Mock-treated *D35S:BOP1-GR* plant showing a wild-type phenotype. D, DEX-induced *D35S:BOP1-GR* plant showing a phenotype similar to *bop1-6D* mutant. E, Comparison of *KNAT6* and *ATH1* transcript levels in the wild type versus *bop1-6D* mutants and mock versus DEX-induced *D35S:BOP1-GR* plants after continuous treatment for 4 weeks. F, Comparison of *KNAT6* and *ATH1* transcript levels in DEX-induced *D35S:BOP1-GR* lines with and without protein synthesis inhibitor CHX. Transcripts were measured after 2 and 4 h of treatment. Bars = 2 cm.
Figure 5. Identification of the genomic region responsible for \textit{ATH1} induction by BOP1.

A to H, Functional characterization of the \textit{ATH1} regulatory region. Representative expression patterns are shown for \textit{D35S:BOP1-GR} plants containing 2(A, C, E, and G) or 3.3-kb (B, D, F, and H) \textit{ATH1p:GUS} reporter genes as diagrammed in I. Promoter activity was monitored by GUS staining after incubation of 10-d-old seedlings or 6-week-old inflorescences for 4 h in mock or 30 µm of DEX solution. Comparison of mock (A–D) and DEX (E–H) shows that expression is up-regulated in the leaves, flowers, and stem of DEX-induced lines for both promoter constructs. Bars = 1 mm. I, Map of the \textit{ATH1} promoter and 59 untranslated region. Black arrowheads mark the 59 ends of genomic fragments used in construction of 2and 3.3-kb \textit{ATH1p:GUS} reporter genes. Predicted consensus binding sites for TGA bZIP factors (Schindler et al., 1992; Izawa et al., 1993; Fode et al., 2008) are shown in relation to fragments amplified by qRT-PCR after ChIP to test for BOP1 localization (horizontal bars). Sites in red (IV and VII) contain A boxes and show enrichment for BOP1. J, Quantification of BOP1-GR enrichment at sites IV and VII in the \textit{ATH1} promoter by qRT-PCR. Anti-GR ChIP was performed using leaves from mock and DEX-treated \textit{35S:BOP1-GR bop1 bop2} plants. Fold enrichment at sites IV and VII is presented as the ratio of DEX versus mock transcript levels after normalization to the unrelated \textit{UBQ5} control sequence. Three biological replicates were quantified to show enrichment at site IV. One biological replicate was quantified to show enrichment at site VII. Three technical replicates were performed for each. Error bars indicate SD.
Figure 6. Quantification of flowering time and meristem identity transcripts in the wild type and mutants.

A, Quantitative analysis of flowering time. Plants were grown under LDs. Date of apex emergence for \textit{bop1 bop2 pny pnf}, \textit{knat6 pny pnf}, and \textit{ath1 pny pnf} mutants is comparable with that of the wild type with minor variations. Lines containing \textit{ath1} flowered slightly earlier (26.7 d) and lines containing \textit{bop1 bop2} flowered slightly later (+3.1 d) than the wild type. *, Significant differences (Student’s t test; P < 0.01). B, Quantitative analysis of meristem identity gene expression. Flowering was induced by shifting plants from SDs to LDs. Apices were harvested on day 37 at the end of 12 LDs. IM identity gene transcripts \textit{SOC1} and \textit{FUL} are expressed at similar levels in Col-0 and \textit{pny pnf} apices. Floral meristem identity gene transcripts \textit{FD}, \textit{LFY}, \textit{AP1}, and \textit{CAL} are significantly lower in \textit{pny pnf} compared with Col-0 apices. Transcript accumulation resumes in \textit{bop1 bop2 pny pnf}, \textit{knat6 pny pnf}, and \textit{ath1 pny pnf} apices. *, Significant differences (Student’s t test; P < 0.05). C, Quantitative analysis of \textit{miR156} and SPL transcript abundance in wild-type and mutant apices. Nonflowering in \textit{pny pnf} correlates with a significant increase in \textit{miR156} abundance at the expense of \textit{SPL3, SPL4, SPL6, SPL9}, and \textit{SPL15} transcripts relative to Col-0 plants. Transcript accumulation in \textit{bop1 bop2 pny pnf}, \textit{knat6 pny pnf}, and \textit{ath1 pny pnf} mutants follows a pattern similar to the wild type, consistent with restored flowering. *, Significant differences (Student’s t test; P < 0.05).
Figure 7. **BOPI** overexpression mimics *pyn pnf* defects in *SPL* transcript accumulation and GA homeostasis.

Plants were grown in continuous light. qRT-PCR was used to assess transcript accumulation in apices and/or internodes. A, Accumulation of *miR156* and *SPL* transcripts in Col-0 and *bop1-6D* internodes. B, Schematic representation of non-13-hydroxylated GA biosynthetic and catabolic pathways in *Arabidopsis* (Hu et al., 2008; Yamaguchi, 2008). Green lettering indicates GA biosynthetic enzymes monitored for transcript accumulation in C and D. Red lettering indicates GA catabolic enzyme monitored for transcript accumulation in C and D. Bioactive GA_4_ is indicated in bold. Inactive GA metabolites shown on right. CDP, *ent*-Copolyal diphosphate; CPS, *ent*-copalyl diphosphate synthase; GGDP, geranylgeranyl diphosphate; KAO, *ent*-kaurenoic acid oxidase; KO, *ent*-kaurene oxidase; KS, *ent*-kaurene synthase. C and D, Accumulation of GA pathway transcripts in *pyn pnf* apices and *bop1-6D* apices and internodes. E, *pyn pnf* and *bop1-6D* plants treated with 100 µm of GA_3_ or a mock solution. F, Flowering time and plant height of Col-0 and *bop1-6D* plants treated with 100 µm of GA_3_ or a mock solution. *, Significant differences (Student’s t test; *P* < 0.05).
Figure 8. Transcript profiling of floral repressor genes in *bop1-6D* and *pny pnf* mutants.

A, Floral repressor genes differentially expressed in *bop1-6D* compared with Col-0 internodes according to microarray experiment (“Materials and Methods”). B, Repressor transcript profile of *bop1-6D* and *pny pnf* mutants quantified by qRT-PCR. No differential expression was observed for FLC transcript. Transcripts encoding AP2-like TOE2 and SMZ repressors and AP2/ERF Dehydration-responsive Element (DRE)-binding Protein-like TINY, DDF1, and DDF2 repressors were differentially up-regulated in agreement with A. *, Significant differences (Student’s t test; P < 0.05).
A, JA-related genes differentially expressed in bop1-6D compared with Col-0 internodes identified by microarray experiment (“Materials and Methods”). B, Schematic representation of the JA biosynthetic pathway in Arabidopsis (Park et al., 2002; Wasternack and Hause, 2013). Red lettering indicates transcripts investigated by qRT-PCR in C. Linolenic acid is released from membrane lipids by a lipolytic enzyme (DEFECTIVE IN ANther DEHISCENCE1 [DAD1]) and converted to allene oxide (12,13-epoxy-octadecanetrienoic acid) by lipoxygenase (LOX) and AOS. One cyclization, one reduction, and three rounds of β-oxidation steps are required in producing JA, which is conjugated to Ile (JA-Ile) in bioactive form (Wasternack and Kombrink, 2010). ACX, Acetyl-CoA oxidase; AOC, allene oxide cyclase; JAR1, JASMONATE RESISTANT1; KAT, L-3-ketoacyl CoA thiolase; MFP, multifunctional protein; OPR3/DDE1, 12-oxo-phytodienoic acid-10,11-reductase3/DELAYED DEHISCENCE1. C, Quantitative analysis of JA biosynthetic gene transcripts in bop1-6D and pny pnf mutants grown under SDs or LDs. *, Significant differences (Student’s t test; P < 0.05). D, Concentration of JA in wild-type tissues compared with bop1-6D, bop1 bop2, and pny pnf mutants (“Materials and Methods”).

Figure 9. BOP1 overexpression increases JA content by transcriptional up-regulation of biosynthetic genes.
Figure 10. Effect of loss or gain of JA content on phenotype of the wild type and mutants.

A to G, Wild-type and pny plants were sprayed daily until maturity with 100 µm of MeJA or a mock solution. A, Mock-treated Col-0 plant. B, MeJA-treated Col-0 plant showing small dark green leaves. C, bop1-6D mutant showing a compact rosette similar to that in B. D, JA-treated Col-0 plants showing pny-like partial loss of apical dominance and short stature. E, JA-treated pny mutant showing enhancement of defects in internode elongation and apical dominance relative to mock control (see G). F, JA-treated pny mutant showing delayed flowering relative to mock control. G, Quantitative phenotypic analysis of wild-type and pny mutant plants treated with MeJA. Plants were grown under LDs. For both genotypes, treatment with MeJA resulted in additional rosette paraclades, indicating loss of apical dominance, reduced height, and delayed flowering. H to J, Effect of aos loss of function on pny pnf and bop1-6D phenotypes. Representative plants are shown. H, pny pnf aos mutant remains nonflowering. I and J, Phenotype of bop1-6D versus bop1-6D aos mutants. A small but highly significant (P < 0.0001) increase in plant height (+1.26 cm) and earlier flowering (21.8 d) are measured in bop1-6D aos compared with bop1-6D control plants. Analysis was performed in a segregating population (n = 100). Bars = 1.5 cm. *, Significant differences (Student’s t test; P,0.05).
Figure 11. Summary and model.

PNY-PNF/STM limit sex expression of BOP1/2 and downstream effector ATH1/KNAT6 to boundary domains flanking the IM. BOP1 acting through an unknown TGA bZIP cofactor directly activates ATH1, whereas promotion of KNAT6 is indirect (red arrow). These products form a module that represses growth, meristem activity, and flowering by increasing JA content by transcriptional promotion of JA biosynthetic genes. Either directly or indirectly (dashed lines), we propose that misexpression of this pathway leads to down-regulation of GA pathway components and repression of the miR156-SPL-miR172 module at one or more nodes in correlation with increased content of associated classes of floral repressors (e.g. DELLA, AP2-like, and AP2/ERF clades). Ultimately, SPL and FD/FT transcripts (not depicted) fail to accumulate, and activation of floral meristem identity genes LFY, AP1, and CAL required for flower initiation is blocked. Internode elongation is also blocked.
5.6 REFERENCE


Khan M, Tabb P, Hepworth SR (2012a) BLADE-ON-PETIOLE1 and 2 regulate Arabidopsis inflorescence architecture in conjunction with homeobox genes KNAT6 and ATH1. Plant Signal Behav 7: 788–792


CJ, Hepworth SR (2012b) Antagonistic interaction of BLADE-ONPETIOLE1 and 2 with
BREVIPEDICELLUS and PENNYWISE regulates Arabidopsis inflorescence architecture. Plant Physiol 158: 946–960


Proveniers M (2013) Sugars speed up the circle of life. eLife 2: e00625


CHAPTER 6 - GENERAL DISCUSSION AND CONCLUSIONS

The studies in this thesis employed large-scale analyses and biophysical methods to study the molecular mechanisms of plant immunity in detail and to move the molecular knowledge towards applications in agriculture. In this part of the thesis, we will discuss an important step of translating the knowledge gained from the model plant *Arabidopsis* to crops in agricultural fields. Such step is to define NPR1 orthologues in crops. Furthermore, future experiments involving proximity ligation assay to study plant immune protein at ultrasensitive level will also be discussed. Finally, conclusions of the entire thesis will be made.

6.1 Defining NPR1 orthologues in crops and the pitfall of percentage sequence identity

NPR1 is found in all crops of commercial significance, whether, like the model plant *Arabidopsis*, they are dicots, such as soybean (Sandhu et al. 2009; Pandey et al. 2011), or monocots, such as rice (Yuan et al. 2007). Functionally, NPR1 are conserved from species to species. For example, *Arabidopsis* NPR1 will function when transformed in rice (Chern et al. 2001). The reverse is also true, where rice (Yuan et al. 2007) or soybean (Sandhu et al. 2009) NPR1 will function in *Arabidopsis* and complement the *npr1* mutation. However, the NPR1 amino acid sequence is not identical in all plants. There is a high sequence diversity among the members of the NPR1 superfamily. Within a plant species, NPR1 is part of a multigene family. For example, in the model system *Arabidopsis*, there are six members in the family, NPR1 to NPR6 (Liu et al. 2005). Two other *Arabidopsis* NPR1-like proteins, NPR3 and NPR4, have been shown to play a role in SA-dependent immunity (Fu et al. 2012). However, these proteins serve to degrade
NPR1 and are therefore negative regulators of immunity. Given the universality of NPR1 and the fact that it has paralogues within a given species, it has become important to identify which NPR1-like gene encodes the real NPR1 protein. This will allow for a faster and more efficient translation of the knowledge garnered from Arabidopsis. Furthermore, the sequence diversity amongst the members of the NPR1 superfamily will lead to a greater understanding of what makes an NPR1, from a secondary structure perspective.

A typical approach to identify an NPR1 orthologue, or any orthologous gene for that matter, is to perform a BLAST search and select the NPR1-like sequence that is the closest in identity to the Arabidopsis NPR1. A more sophisticated approach is to compare the sequence identity of the potential orthologue by performing a pair-wise comparison of the full-length sequences using an alignment software such as Clustal (Larkin et al. 2007; www.clustal.org). Clustal can then be used to create a more visually appealing rendering of the data by producing a ‘phylogenetic’ tree, if more than two sequences are used in a multiple alignment experiment. The data in Table 1 have been produced by extracting all the NPR1-like sequences found in Arabidopsis and soybean using a protein-protein BLAST search with default parameters (blast.ncbi.nlm.nih.gov). The amino acid sequences from the matches were then extracted and subjected to a multiple alignment using ClustalX version 2.1 (Larkin et al. 2007). The percent identity resulting from the pair-wise comparisons, performed by Clustal, is reported in Table 1.

The top section of Table 1 reports on the pair-wise comparison between the six NPR1 family members found in Arabidopsis. One can see that NPR1 is most similar to NPR2, with 63% amino acid sequence identity. NPR3 is most similar to NPR4, with 74%
identity. NPR5 is most similar to NPR6, with 85% identity. Despite the high percent sequence identity between NPR1 and NPR2, NPR2 is not a functional NPR1, since loss-of-function in a single gene (NPR1) is sufficient to abolish PR1 gene activation and SAR deployment (Cao et al. 1997; Ryals et al. 1997).

The bottom section of Table 1 reports on the pair-wise comparison between *Arabidopsis* NPR1 to NPR6 and the seven NPR1-like sequences found in soybean. We chose soybean as a comparative organism because its genome is sequenced and available publicly (www.plantgdb.org). The soybean NPR1-like sequence with the highest sequence identity to *Arabidopsis* NPR1 is Gm09g07440.1, with 52% identity. One could therefore conclude that Gm09g07440.1 is the likely soybean orthologue of NPR1. However, 52% is below the percent identity found between *Arabidopsis* NPR1 and NPR2. Furthermore, *Arabidopsis* NPR2 is also 51% identical to this gene. Therefore, is Gm09g07440.1 an NPR1 or an NPR2 orthologue? Continuing the analysis reveals that Gm09g02430.1 and Gm15g13320.1 are the closest members to *Arabidopsis* NPR3 and NPR4, with percent identity between 61 and 63%. Again, which is the NPR3 or NPR4 orthologue? And so on for the orthologues of NPR5 and NPR6. Interestingly, two different research groups have demonstrated using a combination of genetic complementation tests, in the *Arabidopsis npr1-1* mutant background (Sandhu et al. 2009), and virus-induced gene silencing (VIGS) in soybean (Pandey et al. 2011), that Gm09g02430.1 and Gm15g13320.1 are indeed the orthologues of *Arabidopsis* NPR1 despite being more closely related, in terms of sequence identity, to NPR3 and NPR4. Gm09g02430.1 and Gm15g13320.1 are only 38% identical to *Arabidopsis* NPR1, yet they are the true NPR1 orthologues.
We next performed a ‘phylogenetic’ analysis of the *Arabidopsis* and soybean NPR1-like proteins, which is depicted in Fig. 1. Once again, we used ClustalX version 2.1, which is not regarded as a specialist phylogenetic analysis program, but is the typical go-to software used by non-specialists, including ourselves. These trees are often used by biochemists to identify enzymes with related functions. The tree of Fig. 1 shows three distinct clades, which we identified as NPR1/NPR2, NPR3/NPR4 and NPR5/NPR6. Remarkably, Gm09g02430.1 and Gm15g13320.1, which are the true NPR1 orthologues based on complementation of the *npr1-1* *Arabidopsis* mutant, are in the same clade as NPR3 and NPR4.

It may not be possible to distinguish an NPR1 orthologue from another species simply by comparing their amino acid sequences with that of the *Arabidopsis* NPR1. A functional test is needed in which the NPR1-like genes will be transformed in one of the *Arabidopsis npr1* mutant backgrounds for a complementation test. Alternatively, a loss-of-function mutant in the potential crop NPR1 gene could be used to identify potential NPR1 orthologues by testing the effect of the mutation on the activation of the crop *PRI* gene or the deployment of the crop SA-dependent immunity. However, we do not believe that this can substitute for a complementation test, since a loss-of-function in an NPR1-like gene only indicates that this gene is involved in the activation of *PRI* or in the deployment of SA-dependent immunity. It does not de facto indicate that the gene is an NPR1 orthologue. Finally, the simple analysis presented here indicates that much work is still needed to pinpoint the key amino acids that truly define an NPR1.
6.2 Proximity ligation assay for ultrasensitive plant immune protein analysis

6.2.1 Background of proximity ligation assay

Polymers are fundamental components of all living organism. Cells produce three types of polymers (macromolecules): polysaccharides, proteins and nucleic acids (Lodish et al., 2000). For example, polysaccharides are essential components of plant cell walls and insect skeleton. Nucleic acids and proteins dictate the biological structure and function of all cells. Nucleic acids encode genetic information. Proteins are the main players ensuring the accurate decoding of the genome and executors of the majority of cellular functions. Proper synthesis, execution of function and degradation of proteins ensure proper life cycle of cells. Conversely, any small missteps in protein-related cellular processes can compromise cell fitness and cause detrimental effects, such as diseases.

Due to the importance of proteins, protein detection is a necessary routine procedure used in many fields of fundamental and clinical research and disease diagnostics. The current “gold standard” for protein detection is the enzyme-linked immunosorbent assay (ELISA) (Gijohann and Chad, 2009). Depending on the sensitivity and specificity of the antibodies being used, the detection limit of ELISA ranges from 1pM to 10 pM (roughly $10^7$ molecules in a 60µl drop). However, in basic research, the true concentration of a target protein in situ is often unknown because the protein abundance is below the detection limit of current techniques. In terms of the diagnostic use, the measurement of many protein biomarkers is unfeasible due to the lack of ultrasensitive assays. For example, prostate specific antigen (PSA) is a valid biomarker for prostate cancer recurrence (Gijohann and Chad, 2009). However, serum PSA is
undetectable in patients with recurrent prostate cancer. Therefore, the detection limits of current techniques cannot meet the stringent demands of research or diagnostic needs.

In general, a protein detection assay consists of two steps, biorecognition and signal transduction (Soleymani and Li, 2017). Biorecognition, as the name implies, is the process of target molecule recognition, which requires a sensitive and specific binding of affinity probes to a protein. Currently, two types of molecules are used as affinity probes for protein recognition, antibodies and aptamers. Antibodies are Y-shaped proteins produced by white blood cells. Aptamers are oligonucleotides that are developed through an in vitro selection process (Li et al., 2014). Both antibody and aptamer can be used to specifically bind with a certain protein molecule. Signal transduction is the step in which the presence of a target molecule is converted into a measurable signal. The produced signal should be proportional to the concentration of analyte being measured. Improved performance compared with ELISA in either of or both of biorecognition and signal transduction steps can result in an ultrasensitive protein detection assay. Proximity ligation assay (PLA) achieves ultra-low protein detection through a superior design of the signal transduction step (Fredriksson et al., 2002).

This part of the discussion will focus on the design of PLA. Particularly, we will look at the applications of PLA for in vitro protein detections, in situ PLA for direct visualization of proteins or protein complexes and multiplexed PLA for disease biomarker validation.

6.2.2 PLA

In comparison to protein detection methods, those developed for nucleic acids, such as polymerase chain reaction (PCR), have excellent specificity and sensitivity. The
detection limit of PCR is $10^6$ fold lower than that of ELISA (Gijohann and Chad, 2009). Therefore, transducing the recognition of protein targets into a nucleic acid detection system is a very tempting approach (Li et al., 2014). PLA exploits such strategy to realize ultrasensitive protein detection in homogeneous environments.

Landegran and his colleagues first developed PLA in 2002. In a PLA experiment, the recognition of a protein target, by a pair of proximity probes, leads to the production of a newly ligated DNA template. This template can then be detected by quantitative PCR (qPCR). The conversion of protein detection to the generation of a DNA template is possible because of the design of the proximity probes. Proximity probes are composed of a pair of target-specific affinity probe, each attaching to an oligonucleotide. When two affinity probes simultaneously recognize a target molecule, the free ends of the two attached oligonucleotides are brought sufficiently close. Upon addition of a template DNA (also called connector DNA) and ligase, such proximity allows the two free ends to hybridize to the connector oligonucleotide and to be joined by enzymatic ligation reaction. The detection of a target by a pair of proximity probe results in the production of a newly ligated DNA, which can be amplified by qPCR. The unreacted proximity probes remain silent. Because the amplifiable signal can only be generated after two simultaneous and proximate recognitions of the same target molecule, excessive assay reagents will not lead to any detectable signals. As such, no separation and washing steps are required in a PLA experiment.

PLA was used to detect the homodimer of platelet-derived growth factor B-chain (PDGF-BB) (Fredriksson et al., 2002). PDGF specific aptamers were used as affinity probes. Extending PDGF aptamers with additional sequences at either the 5’ or 3’ end
generated a pair of proximity probe. When two probes with oligo extensions at two different ends bound with the same target molecule, a series of signal transduction events were triggered and an amplified signal can be detected (Figure 2). Using PLA, the authors could detect 24,000 PDGF-BB molecules in a 5µl solution. The detection limit of PLA was about 1000 fold lower than was possible by an ELISA assay quantifying the same protein.

6.2.2.1 Deep inside a PLA assay

There are several things that need to be considered in the design of a PLA experiment. First, two affinity probes must be available for the target of interest. Both aptamers and antibodies can be used as affinity probes in a PLA experiment (Gullberg et al., 2004). In addition, three aspects about the design of the connector DNA are worth noting. First, in order to prevent the connector DNA from giving rise to ligation-independent amplification products by acting as a primer, a short hybridizing length that does not pair with the proximity probes need to be included at the 5’ and 3’ of the connector DNA. Second, the hybridizing length of the connector DNA should be no longer than 20bp to avoid false positive PCR products caused by target-independent ligation products. Finally, to ensure the efficiency of the ligation, the connector DNA needs to be used in large molar excess over the proximity probe (about 20,000-fold higher connector concentration was used in the PDGF-BB experiment).

Despite the greatly improved low detection limit, PLA had a narrow dynamic range in the measurement of PDGF-BB (three orders of magnitudes). The dynamic range of a protein detection assay refers to the range of antigen concentrations that can be measured accurately (Mikkelsen et al., 2016). The upper limit of PLA’s dynamic range is
unavoidably restrained by the “proximate” nature of the assay. In a PLA experiment, in order to generate signals, two probes are required to bind with the same target molecule. This works well when the probes concentration are significantly higher than the target concentration. However, when the target concentration rise to a certain level, target molecules start to compete with each other to bind with the probes. Less and less two probes – one-target complexes can form. This will results in inaccurately low results. Such phenomenon is known as “the hook effect”, which is also a common problem of the “sandwich” ELISA assays (Miller. 2004). The lower detection limit of PLA is restricted by the problem of low signal to noise ratio. For example, no significantly different readout was generated in a PLA experiment whether using 20,000 molecules of PDGF-BB or no molecules. The unintended ligation products generated from the target-independent hybridization between unbound probes and connector DNA are the causes of the false positive results.

Nevertheless, PLA successfully achieved ultrasensitive protein detection in homogenous solutions by converting the detection of protein into more sensitive nucleic acids detection. PLA has indisputably lower detection limit compared to ELISA for the same protein. Moreover, PLA has been shown to be a highly elastic technology that can be applied to meet various needs of protein detections, as it will be reviewed in the following sections.

6.2.3 Ultrasensitive protein analysis

The proper function of a protein is determined by four aspects of its behavior: concentration, localization, interactions with other molecules and post-translational modifications. Ultrasensitive analyses in all these four aspects are in demand for both in
vitro and in situ studies. In vitro experiments allow characterization of a protein’s function in controlled settings, for example, comparing binding affinity of two different drugs to one particular target. In situ studies offer insights into proteins behavior in their native environments, such as, studying drug perturbation of protein-protein interactions in a specific cell type. PLA has been successfully transformed into both in vitro and in situ protein detection methods. Moreover, PLA holds great potential for the measurement of large set of proteins in one sample. Here, we will discuss three types of PLA applications in detail: in vitro and in situ ultrasensitive protein analyses and multiplexed PLA.

6.2.3.1 Detection of protein interactions in vitro

PLA was originally demonstrated to be an ultrasensitive protein quantification technology in vitro. However, due to the requirements of the two probes-binding events in PLA, it can be easily transformed to study protein-protein interactions or protein-other macromolecule interactions by designing each probe to specifically recognize one of the two binding partners. Moreover, because of PLA’s ultra-low detection limit, it can be used to study protein interactions in depth in vitro. Methods for detecting protein-DNA interactions and high-throughput drug screening for inhibitors of protein-protein interactions have all been developed based on PLA (Gustafsdottir et al., 2007; Gustafsdottir et al., 2008).

6.2.3.1.1 In-depth characterization of DNA-binding proteins’ sequence specificity

The most intensively studied sequence-specific DNA-binding proteins are transcription factors. A transcription factor is a protein that controls the rate of DNA being transcribed into messenger RNA by binding to a specific DNA sequence (Lodish et al., 2000). Identifying binding sequence of transcription factors has profound clinical
importance for both animals and plants. This is because many cellular functions and the activation of the immune system often require accurate actions of transcription factors. Many diseases, particularly cancer, happen because of malfunctions of transcription factors. Genome-wide location map of DNA-binding proteins from multiple organisms, including human, yeast and Arabidopsis, have been identified using chromatin-immunoprecipitation followed by microarray or sequencing analyses (ChIP-chip/seq) technology (Ren et al., 2000; Lee et al., 2002; Weinmann, 2002; O’Malley et al., 2016). ChIP-chip/seq provided valuable information on genome-wide DNA-binding sites of transcription factors. However, these techniques have low resolution: DNA binding sites identified by CHIP-chip/seq are typically around 100-300bp (Landt et al., 2012). Even with the help of bioinformatics prediction software, results extracted from ChIP-chip/seq experiments must be confirmed by a gel electrophoresis based method, the electrophoretic mobility shift assay (EMSA). This method can narrow down DNA-binding sequence to high resolution (shorter than 30bp) (Boyle et al., 2009). However, EMSA is a relatively labor-intensive procedure, which requires large amount of protein and reagents.

Gustafsdottir et al established an in vitro method for specific and sensitive protein-DNA interaction measurement base on the proximity ligation assay. To adapt PLA for protein-DNA interaction, one of the proximity probes was designed to be a partly double-stranded oligonucleotide with a single-stranded 3’ extension. The other one kept the standard design, which is an antibody attached to a DNA strand with a free 5’ end. When the protein bound with the double stranded sequence, the two free oligonucleotide ends of the proximity probes were ligated assisted by a connector DNA
and a ligase. Thus an amplifiable signal was generated and afterward detected by qPCR. The authors successfully used this method to test sequence specificities of three transcription factors, tumor protein p53, hepatocyte nuclear factor HNF-1α and upstream stimulatory factor (USF1). PLA was used to measure protein-DNA interactions in a quantitative and highly reproducible manner. Moreover, this method can be performed in fewer than 4 h with a very low reagent and sample consumption.

6.2.3.2 In situ protein visualization

*In vitro* analysis allows qualitative and quantitative examination of protein behaviors in depth. However, *in situ* protein detection is of equal importance as it permits observing a protein’s behavior in its natural environment. *In situ* analysis is the perfect test ground for what we have learned about a protein *in vitro*.

Because PLA can transduce protein recognition into the production of an amplifiable nucleic acid, a variety of techniques that are available for nucleic acid detection can be coupled with PLA. Rolling circle amplification (RCA) is a process of unidirectional nucleic acid replication that can rapidly synthesize multiple copies of a single-stranded, circular DNA molecule (Ali et al., 2014). The product of a RCA reaction is a randomly coiled single-stranded DNA. Hybridizing probes labeled with fluorescent molecules can be easily designed and used to detect a RCA product under a fluorescent microscope, which make RCA ideal for cell imaging. By combining RCA with PLA, one can achieve direct visualization of proteins and protein complexes at *in situ* locales (Söderberg et al., 2006).

6.2.3.2.1 In situ PLA, a disruptive technology

Prior to *in situ* PLA, two types of techniques were mainly used to study protein-
protein interactions *in situ*, including protein pull-down related systems, such as coimmunoprecipitation (co-IP) and fluorescence based assay, such as bimolecular fluorescence complementation (BiFC) (Golemis and Adams, 2005). In a co-IP experiment, the protein of interest is precipitated using a specific antibody attached to a solid support. During this precipitation process, its interacting partner will be pulled down together. The identity of the interacting partner can be subsequently confirmed by western blots. BiFC involves expressing fusion proteins made of complementing fluorescence protein fragments and postulated interacting proteins *in situ*. Reconstituted fluorescence signals are used as reporters for protein interactions. Neither co-IP type nor BiFC type techniques can visualize endogenous proteins interaction (a detail comparison of *in situ* PLA, co-IP and BiFC is summarized in Table 2). *In situ* PLA is uniquely suited to visualize interactions of endogenous proteins directly in fixed cells and tissues. Due to the distinctive advantages of *in situ* PLA, it has been widely used in basic and clinical research (Figure 3).

6.2.3.2.2 Visualization of protein-protein interactions *in situ*

Landegran and his colleagues adopted proximity ligation for cell imaging in 2006. In order to couple RCA with PLA, proximate bindings of two probes to one protein target need to give rise to the production of a circular DNA that can be used as a template in RCA reaction. Two free oligonucleotide ends of the proximity probes were designed to be templates for the circulation of a connector DNA by enzymatic ligation. Upon binding to the same target or target complex, the proximity between a pair of probes led to the creation of a circular DNA. Neither of the two proximity probes can result in circulation of a connector DNA on its own. The circularized DNA remained attached to the
proximity probes. After addition of phi29 DNA polymerase, a polymerase with exceptional strand displacement activity, one of the oligonucleotides served as a primer for the RCA reaction. The other probe’s extending function was blocked by addition of three mismatched, exonuclease-resistant 2’O-methyl RNA nucleotides at the 3’ end. As such, phi29 catalyzed replications of the circular DNA using one of the proximity probes as a primer, while unwinding the hybridizing oligo from the DNA circle. A one hour RCA reaction generated a randomly coiled, single-stranded DNA composed of 1000 copies of the circular connector DNA (Figure 4).

*In situ* PLA was used to detect interactions of endogenous transcription factor c-Myc and its binding partner Max. c-Myc is an proto-oncoprotein. Overexpression of c-Myc is observed in many cancers (Pelengaris et al., 2002). Unregulated c-Myc leads to uncontrolled cell growth and results in the formation of cancer. Thus, c-Myc is viewed as a promising target for anti-cancer drugs (Tansey, 2014). *In situ* PLA was shown to be able to detect c-Myc/Max interactions in various cell types and clinical-relevant tissue samples. Furthermore, *in situ* PLA can also be modified to detect multi-protein complexes by introducing a third proximity probe that is required for connector DNA circularization, as it was demonstrated through the detection of RNA-polymerase, c-Myc and Max interactions.

Remarkably, drug perturbations of c-Myc/Max heterodimerizations could also be monitored using *in situ* PLA in a sensitive and quantitative fashion. Based on this method, a high content screening assay for inhibitors of protein interactions in primary cells was developed using proximity ligation (Leuchowius et al., 2010).
6.2.3.2.3 Visualization of posttranslational modification in situ

Posttranslational modification of proteins can also be visualized by in situ PLA (Jarvius et al., 2007).

Platelet-derived growth factor receptor β (PDGFRβ) is a transmembrane receptor kinase that can bind to the cytokine PDGF-BB. Binding of PDGF-BB to PDGFRβ leads to the activation of several signaling pathways that promote cell proliferation, motility and survival (Heldin et al., 1998). The very first step of these signaling pathways is PDGFRβ autophosphorylation triggered by the perception of PDGF-BB. Endogenous tyrosine phosphorylated PDGFRβ was detected in immortalized human foreskin fibroblasts cells (BJ hTert) and human scar tissue.

One notable thing of this application is that the detection of phosphorylated PDGFRβ was achieved through a generalized proximity ligation method. Secondary antibodies with attached DNA strands were used as proximity probes. In the case of the phosphorylated PDGFRβ detection, two primary antibodies raised in two different species were first incubated with fixed cells or tissues. One antibody specifically recognized the C-terminal part of the PDGFRβ and the other bound to the phosphorylated tyrosine751 of PDGFRβ. A pair of secondary antibody, each recognizing one of the primary antibodies was added next. Only when both primary antibodies were bound to the target and in turn recognized by the proximity probes, could the circularization of the connector DNA and the subsequent RCA reaction be triggered. Endogenous phosphorylated PDGFRβ were detected in cells and tissues using secondary antibody-proximity probes with great sensitivity and specificity.

This generalized method allowed for sensitive and specific protein detection.
without conjugating primary antibodies to oligonucleotides. Selections of ready to use proximity probes, composed of secondary antibodies with attached DNA strands, are available for purchase at Sigma-Aldrich. With the help of the commercially available proximity probes, any protein target with an available pair of primary antibody raised in two different species has the potential to be analyzed by *in situ* PLA. Such convenience simplifies adaptions of *in situ* PLA to research from various backgrounds. Generalized *in situ* PLA has been used to study transcription factors, receptor proteins and cell signaling proteins in animal cells and tissues, yeast cells and plant tissues (Xu et al., 2014; Taylor et al. 2012; Jarvius et al., 2007; Acharya et al., 2016)

### 6.2.3.2.4 Visualization of newly synthesized target protein *in situ*

A recently developed application of *in situ* PLA (Dieck et al., 2015), which permits direct visualization of a newly synthesized target protein, could also contribute to the booming of new research areas.

A cellular proteome is a dynamic reservoir instead of a still pool of proteins. Recent research on a major protein degradation pathway of eukaryotic cells, autophagy, has further demonstrated the importance of protein dynamics. Proper degradation of protein is crucial to the preservation of human fitness. It is involved in critical processes such as aging, eliminating invading pathogens and fighting neurodegeneration and cancer (Galluzzi et al., 2017). To recognize the importance of autophagy, Dr. Ohsumi has been recently awarded the Nobel Prize in physiology or medicine for his discoveries of autophagy mechanisms. In contrast to protein degradation, the other side of the protein dynamics equation, protein synthesis, has not been intensively studied.

Traditionally, newly synthesized proteins are detected *in situ* using pulse-chase
analysis (Takahashi and Ono, 2003). In a pulse-chase experiment, cells are first exposed to radioactive amino acids for a short period of time (pulse labeling) and then to an excess amount of unlabeled amino acids (chase). The radioactive protein of interest is isolated from other cellular proteins by immunoprecipitation and Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then quantified by scintillation counting.

Azidohomoalanine (AHA) was developed as a replacement of radioactive molecules in pulse-chase analysis (Dieterich et al., 2006). AHA is a noncanonical amino acid that can be use as a surrogate for methionine. AHA can be fed to cultured cells and incorporated into newly synthesized proteins during translation. Incorporation of AHA introduces an azido moiety. This allows newly synthesized proteins to be tagged by click chemistry, purified using affinity purification and identified by mass spectrometry. Noncanonical amino acid also enables the visualization of newly synthesized proteome in situ (fluorescent noncanonical amino acid tagging, also known as FUNCAT) (Dieterich et al., 2010). Upon incorporation of AHA to newly synthesized protein, fluorescent tags can be added to AHA via click chemistry to allow visualization of newly synthesized proteome (Figure 5a). Alternatively, a biotin-based tag is added and the AHA incorporated proteome is visualized using fluorescent anti-biotin antibody. Although AHA can be used to detect changes and locations of protein synthesis and evaluate the spatial fate of protein, AHA incorporation requires relatively long pretreatment time (minutes to hours) due to rate limiting AHA uptake and activation by the methionyl tRNA synthetase processes (Dieck et al., 2015).

Puromycin is another chemical that can be used as a metabolic label to quantify
and visualize nascent proteome (Schmidt et al., 2009). Puromycin is an antibiotic protein synthesis inhibitor that can be incorporated into polypeptide chains, to terminate translation and to release truncated proteins. When used at a low concentration, puromycin incorporation can be used to quantify newly synthesized proteome in situ and to visualize proteome using anti-puromycin antibody (Figure 5b). Incorporation of puromycin is significantly faster than AHA (seconds to minutes) (Dieck et al., 2015). However, puromycin causes premature termination of the translation process, which results in the production of truncated protein. Thus, puromycin is not suitable to study the spatial fate of proteins.

AHA and puromycin can compensate each other when being used in investigating newly synthesized proteome. However, there is a big blank spot in the area. That is a specific newly synthesized protein cannot be visualized through neither of the two methods.

The double recognition characteristics of PLA come perfectly in play again in the case of specific newly synthesized protein visualization in situ. Incorporation of PLA to both FUNCAT and puromycylation based metabolic labeling (FUNCAT-PLA and Puro-PLA) were demonstrated with the detection of newly synthesized Calcium/calmodulin-dependent protein kinase type II alpha chain (Camk2a) in neurons (Dieck et al., 2015). For the FUNCAT-PLA, classic pulse-chase experiments using AHA were first performed. A biotin-based tag is then added to incorporated AHA by click chemistry (Figure 5c). The specific recognition of a newly synthesized Camk2a is achieved using two primary antibodies; one recognized the tagged biotin and the other specifically bound on Camk2a. After that, a generic in situ PLA method was performed. Two secondary
antibodies coupled to two different oligonucleotides were added and subsequent ligation and RCA reaction were triggered. The detectable signal was generated by the proximity of “newly synthesized” (represented by biotin) and “specific” (represented by anti-Camk2a) recognitions of a Camk2a. Puro-PLA can be performed in a similar fashion using anti-puromycin primary antibody. Visualization and quantification of newly synthesized Cambk2a in neuron cells were accomplished through both FUNCAT-PLA and Puro-PLA.

In addition to visualization and quantification, FUNCAT-PLA was used to determine the half-life of a protein, track its spatial movements inside the cell and measure synthesis in a given time interval (Dieck et al., 2015).

6.2.3.3 Multiplexed PLA

PLA is at a unique niche position wherein protein recognition creates the generation of a distinct and much more versatile molecule, nucleic acid. Due to the convenience of designing and manufacturing unique DNA sequences, DNA has been used as “barcodes” to label genetic samples in various research fields. When one studies the evolution of different species, a short genetic marker in an organism’s DNA can be used as a DNA barcode to identify a certain genome as belonging to a particular species (Herbert et al., 2003). In multiplexed next generation sequencing, individual "barcode" sequences are added to each sample so they can be multiplexed in the sequencing step, but distinguished and sorted during data analysis (Hardenbol et al., 2003). Similar to these applications, attaching different “signature” oligo sequences to distinct affinity probes that are specific to different proteins can allow detections of multiple proteins in one experiment.
To further exploit PLA’s potentials in detecting disease biomarkers, Fredriksson et al developed a multiplexed protein detection method for cancer biomarker validation based on proximity ligation. By attaching unique identifiers to antibodies detecting different proteins, the authors achieved accurate measurements of 6 proteins in a 1µl human plasma sample.

Fredriksson et al demonstrated the use of multiplexed PLA in cancer biomarker validation. However, multiplexed PLA also holds huge promise in fundamental research. Many important regulators of cells control more than one cellular process, for example, *Arabidopsis* transcription factor BRASSINOSTEROID RESISTANCE 1 (BZR1) is the main factor that regulates gene expression in response to the plant growth hormone brassinosteroids (He et al., 2005). Yet, BZR1 is also known to control multiple other systems by interacting with different additional transcription factors. For example, in response to light and temperature, BZR1 interacts with phytochrome-interacting factor 4 (PIF4) and they together bind on more than 2000 genes’ promoters (Oh et al., 2012). While under other circumstances, BZR1 also regulates the expression of immunity related genes by interacting with another transcription factor WRKY40 (Lozano-Duránet et al., 2013). The specific processes controlled by BZR1 at a given time are dependent on its interacting partners. This suggests that the function of BZR1 is determined by allocation of this master regulator to its binding partners. However, the dynamics of BZR1-PIF4 and BZR1-WRKY40 interactions have never been studied given the difficulties of measuring multiple protein interactions in one sample. Multiplexed PLA, in theory, can make investigations of multiple protein interactions in one small amount of cell extract possible. Such multiplexed detection can provide valuable information on how cells
allocate a particular multitasked protein to execute different functions at a given time and under a given condition.

6.2.4. Final thoughts regarding PLA

The development of PLA represents a strong attempt to diminish the significant detection limit gap between proteins and nucleic acids detections. Over the years, the applicability of PLA has been demonstrated in a variety of publications. Some PLA applications, such as in vitro protein interaction detection, stood out because of improved sensitivity and specificity, which allowed more in depth protein characterization. Other applications, such as in situ protein detections, made new frontiers of science much more accessible and permitted researchers to explore crucial aspects of protein function that were previously difficult to visit. DNA reporters-based multiplexing capacity was another neat add-on advantage to all PLA based protein detections. There is no doubt PLA -based ultrasensitive protein detection methods can bring a whole ultrasensitive and quantitative revolution to protein analyses in plant immune system.
6.3 Conclusions of the thesis

Using the larger-scale methods developed or adapted in this thesis, intriguing links between important immune regulators are discovered. TGA1/4 are found to antagonize the brassinosteroids controlled basal immunity through WRKYs and antagonize the control of SA on a set of systemic acquired resistance genes through GRX480. In addition, a biophysical method, DSF, brought us a step closer from translating existing molecular knowledge on NPR1 to crop protection strategies. Finally, the gene encoding an important developmental and flowering transcription factor, \( ATH1 \), was shown to be a direct target of NPR family protein, BOP1 (NPR6). Such discovery would suggest that NPR1 is not the only transcriptional regulator in the NPR family.

Collectively, the studies presented in this thesis are a gateway for the new directions to study the plant immune system, which will be geared towards understanding its integration with other systems. Results from this thesis suggest that the regulation of plant immunity is a multilayered, interconnected and dynamic process. In addition, the large scale and biophysical methods exploited in this thesis have been shown to be useful for studying the complex molecular mechanism of the plant immune system. We anticipate that those methods could be widely used in other research fields.
Figure 1. Phylogenetic tree of *Arabidopsis* and soybean NPR1-like proteins.

The tree is divided into three clades, NPR1/NPR2, NPR3/NPR4 and NPR5/NPR6, based on the *Arabidopsis* NPR1-like proteins. NPR1 and the true soybean NPR1 orthologues appear inside white oval shapes. See text for more details on the figure.
Figure 2. Schematic representation of PLA for *in vitro* protein detection

Figure made according to the experimental designs of Fredriksson et al. **Proximity probe design**, Green: aptamers; Red and blue: two different oligonucleotide extensions. **Biorecognition**, Blue: target protein. **Signal transduction**, Black: connector DNA; Yellow: DNA ligase; Orange: qPCR products.

Figure 3. Number of citations of Söderberg et al from 2006 to 2016

Citation numbers from the Google Scholar search engine.
Figure 4. Schematic representation of *in situ* PLA

Figure made according to the experimental designs of Söderberg et al. **Proximity probe design**, Green and bright blue: two different antibodies, each specifically recognizes one of the binding partners; Red and dark blue: two different oligonucleotide extensions, red is the non-extending oligo, blue can be used as a primer in RCA reaction. **Biorecognition**, Green and blue: interacting target proteins. **Signal transduction**, Black: connector DNA; Yellow: DNA ligase; Orange: RCA product.
Figure 5. Chemical structures of agents used in visualizations of newly synthesized proteins

(a) AHA and two fluorescent tags stuctures used inFUNCAT (Dieterich et al., 2010). (b) Puromycin structure (Schmidt et al., 2009). (c) The biotin based tag structure used in FUNCAT-PLA (Dieck et al., 2015).
Table 1. Soybean orthologues of the *Arabidopsis* NPR identified by percent identity and complementation.

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Table 2. Comparison of in situ PLA, co-IP and BiFC.

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6.4 REFERENCE


Pelengaris, S., Khan, M., & Evan, G. (2002). c-MYC: more than just a matter of life and


