Early Signaling in plant immunity

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the degree Doctor of Philosophy

in

Biology

by

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2013
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Chair

University of California, San Diego

2013
DEDICATION

This dissertation is dedicated to all my friends and family who were supportive during my graduate studies. It is also dedicated to all the students, colleagues and my committee members who contributed to the success of this research.
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<td>1</td>
<td>AGO4: ARGONAUTE 4</td>
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<td>2</td>
<td>AgriGO: GO Analysis Toolkit and Database for Agricultural Community</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>APY1: APYRASE 1</td>
<td></td>
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<tr>
<td>4</td>
<td>APY2: APYRASE 2</td>
<td></td>
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<tr>
<td>5</td>
<td>Apyrase: ATP-diphosphohydrolases</td>
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<tr>
<td>6</td>
<td>ARF-GEF: adenosine diphosphate ribosylation factor guanine nucleotide exchange factor</td>
<td></td>
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<tr>
<td>7</td>
<td>ATGDI2: RAB GDP DISSOCIATION INHIBITOR 2</td>
<td></td>
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<tr>
<td>8</td>
<td>AtMIN7: HOPM INTERACTOR 7</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>ATP: Adenosine triphosphate</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>BAK1: BRI1-ASSOCIATED KINASE 1</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>BES1: BRI1-EMS-SUPPRESSOR 1</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>BIN2: BRASSINOSTEROID INSENSITIVE 2</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Botrytis: Botrytis cinerea (also B.cinerea)</td>
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<tr>
<td>14</td>
<td>BRI1: BRASSINOSTEROID INSENSITIVE 1</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>BRZ1: BRASSINAZOLE-RESISTANT 1</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>BSU: BRI1 SUPPRESSOR 1</td>
<td></td>
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<td>17</td>
<td>BTH: benzo-(1,2,3)-thiadiazole-7-carbothioic S-methyl ester</td>
<td></td>
</tr>
</tbody>
</table>
Col-0: Wildtype Columbia ecotype plants

COR: coronatine

CXIP1: CAX INTERACTING PROTEIN 1

CYP86A2: CYTOCHROME P450 86A2

DC3000: Pseudomonas syringae pv. (Pto) DC3000

DNA: Deoxyribonucleic acid

Dpi: days post inoculation

EDA10: EMBRYO SAC DEVELOPMENT ARREST 10

ELF6: EARLY FLOWERING 6

ENTPD1: ECTONUCLOSIDE TRIPHOSPHATE DIPHOSPHOYDROLASE 1

ETI: effector triggered immunity

FLC: FLOWERING LOCUS C

FLS2: FLAGELLIN-SENSITIVE 2

GDP: guanosine diphosphate

GO term: Gene ontology term

GPAT4: GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 4

GPAT8: PROBABLE GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 8

GTP: guanosine triphosphate
Hpa: *Hyaloperonospora arabidopsidis* (also *H.arabidopsidis*)

HR: hypersensitive response

IR: induced resistance

IRK: INFLORESCENCE AND ROOT APICES RECEPTOR LIKE KINASE

JA: jasmonic acid

LAR: local acquired resistance

LHCB1: LIGHT HARVESTING CHLOROPHYLL B-BINDING PROTEIN 1

LHCB2: LIGHT HARVESTING CHLOROPHYLL B-BINDING PROTEIN 2

LHCB3: LIGHT HARVESTING CHLOROPHYLL B-BINDING protein 3

LHCII: light-harvesting complex II

LRR: leucine rich repeat

MAMPs: microbe-associated molecular patterns

mRNA: messenger RNA

NBS: nucleotide binding site

NPH3: NON-PHOTOTROPIC HYPOCOTYL 3

NPR1: NONEXPRESSER OF PR GENES1

NPR3: NPR1-LIKE PROTEIN 3

NPR4: NPR1-LIKE PROTEIN 4
OPP: oxidative pentose phosphate pathway

P2: NADP-DEPENDENT ALKENAL DOUBLE BOND REDUCTASE P2

PAMPs: pathogen associated molecular patterns

PER63: PEROXIDASE 63

PHOT1: PHOTOTROPIN 1

PKP-ALPHA: PLASTIDIAL PYRUVATE KINASE 1

PR: PATHOGENESIS RELATED

PRR: Pattern recognition receptor

PRXCB: PEROXIDASE CB

PTI: PAMP triggered immunity

R proteins: RESISTANCE proteins

RAT5: RESISTANT TO AGROBACTERIUM TRANSFORMATION 5

REF6: RELATIVE OF EARLY FLOWERING

RIN4: RPM1-INTERACTING PROTEIN 4

RNA: Ribonucleic acid

ROS: reactive oxygen species

RPM1: RESISTANCE TO P. SYRINGAE PV MACULICOLA 1

SA: salicylic acid
SAR: systemic acquired resistance

TAIR: The Arabidopsis information resource

TCA: tricarboxylic acid cycle

Ws: Wassilewskija ecotype

WT: wild-type

ZAP1: ZINC-DEPENDENT ACTIVATOR PROTEIN-
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Parts of chapter 1 and chapter 2 of this dissertation are being prepared for publication. The co-authors are Zhouxin Shen, Michelle Lee, Earl S. Kang, Nuria Sanchez- Coll, Jeff Dangl and Steven P. Briggs. The dissertation author is the primary investigator and author of this paper titled “Proteome Analysis of Early Signaling in Plant Defense against Pathogen Infection”.

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ABSTRACT OF THE DISSERTATION

Early Signaling in Plant Immunity

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Professor Steve P. Briggs, Chair

During pathogen infection, plants recognize microbial molecules known as pathogen associated molecular patterns (PAMPs) by surface localized pattern recognition receptors (PRRs), these results in physiological changes that limit pathogen growth in a process known as PAMP-triggered immunity (PTI). Recognition of PAMPs triggers the production of the phytohormone salicylic acid (SA), which is important for defense against biotrophic pathogens
and requires redox regulated NONEXPRESSER OF PR GENES1 (NPR1), a master regulator of SA-mediated defense. Although there is significant research on the transcriptional changes related to defense signaling, there is limited information on the early defense related protein changes that occurs before major transcriptional changes. Here we present the mass spectrometry result of early changes in protein abundance, and phosphorylation of Arabidopsis thaliana plants treated with defense elicitor BTH. We observed that 48 proteins changed in abundance, and 43 in phosphorylation following BTH treatment. We also analyzed the changes in abundance in NPR1 mutant (npr1-1) and observed that 43 proteins changed in abundance.

We characterized the roles of 9 of the observed proteins in defense against three pathogens Hyaloperonospora arabidopsidis (Hpa), Pseudomonas syringae pv. (Pto) DC3000, and Botrytis cinerea. Our results reveal the novel role of 2 proteins in defense against Pto DC3000, 5 proteins in defense against Hpa and 3 proteins in defense against botrytis. We further analyzed the BTH induced proteome changes observed in our mass spectrometry results of the total proteome, phosphoproteome and npr1-1 proteome with STRING, an online database of known and predicted protein interactions. The predicted networks created from STRING were used in combination with our infection bioassay results to predict the roles of our proteins in known and novel defense networks. Our study emphasizes the strength of mass spectrometry as a tool to discover proteins with observable phenotypes and to predict novel protein networks.
CHAPTER 1

EARLY SIGNALING IN PLANT IMMUNITY

Introduction

Plant pathogens such as *Pseudomonas syringae* plague plants worldwide, resulting in damaging effects such as specks, spots, blights, wilts, galls, cankers and death. These symptoms affect the quality of agricultural crops and can have serious health and economic consequences (Abramovitch et al., 2006).

![Diagram of stomata closure](image)

**Figure 1.1:** Stomata closure is an important part of early response that limits pathogen invasion. (A) Leaf Epidermis consists of closed and opened stomata. (B) PAMP recognition leads to rapid closure of plant stomata (C) *Pseudomonas syringae* produce inducible COR in the apoplast or surface of the plants to reopen closed stomata and increase the number of bacterial invasion sites.

The Plant epidermis is the first line of defense against pathogens as it constitutes the outermost protective layer of the plant. Stomata, which are microscopic pores surrounded by guard cells
located at the leaf epidermis, play an important role in this first line of defense. Besides the stomata’s role in carbon dioxide uptake and transpiration, they also limit bacterial invasion by closure within 2hrs of bacterial infection (Melotto et al., 2006; Melotto et al., 2008). Bacterial pathogen *Pseudomonas syringae* take advantage of this by producing inducible coronatine (COR) in the apoplast or surface of the plants to reopen closed stomata and increase the number of sites for bacterial invasion (Figure 1.1) (Melotto et al., 2008).

PAMP-triggered immunity (PTI) and effector- triggered immunity (ETI) are defense responses mounted by plants to fight against pathogen infection. PTI is triggered by molecular patterns that are common to entire classes of pathogens known as pathogen associated molecular-patterns (PAMP); an example is flagellin. Upon exposure to PAMPs, plants undergo a series of changes including cell wall lignification, production of reactive oxygen species (ROS), calcium burst, production of phytoalexins and transcriptional reprogramming (Hahn, 1996; Lamb and Dixon, 1997; Hammerschmidt, 1999; Jacobs et al., 2003; Tao et al., 2003; Bindschedler et al., 2006; Torres et al., 2006; Segonzac et al., 2011) These defenses serve to limit the extent of pathogen infection (Figure 1.2A).

As a counteraction to PTI, pathogens use the type III secretion system to inject effector molecules that neutralize plant defenses by targeting key members of the PTI pathway and downstream pathways. This mechanism is also used by animal pathogens (Kim et al., 2005; Abramovitch et al., 2006; Dardick and Ronald, 2006) Plants recognize the action of effector molecules by Resistance (R)-proteins, typically nucleotide binding site leucine rich repeat (NBS-LRR) proteins, which are part of the ETI defense response (Takken et al., 2006). R proteins trigger cell death at the site of infection in a process known as hypersensitive response (HR); this prevents further spread of disease (Figure 1.2C). Absence of R-proteins results in pathogen infection and growth (Figure 1.2B) (Bent and Mackey, 2007). An example of R-protein mediated response is the R-protein RESISTANCE TO P. SYRINGAE PV MACULICOLA 1 (RPM1)
recognition of the avrRpm1 effector molecule activity. AvrRpm1 acts by inducing phosphorylation of RPM1-INTERACTING PROTEIN 4 (RIN4), which is a protein involved in PTI. The presence of the RPM1 leads to HR and reduced pathogen growth and resistance, on the other hand, its absence results in susceptibility to infection. Recognition of pathogens by either PTI or ETI leads to systemic acquired resistance (SAR). This defense is activated throughout the entire plant resulting in increased resistance to secondary infection (Belkhadir et al., 2004; Kim et al., 2005) SAR and local acquired resistance (LAR) both qualify as forms of induced resistance (IR).

Figure 1.2: PTI and ETI. (A) Plant pattern recognition receptor (PRR) recognizes the flagellin (PAMP) triggering PAMP-triggered immunity (PTI). (B) Absence of effector-specific plant R protein results in susceptibility to infection. (C) Effector-triggered immunity (ETI) is triggered by R protein recognition of effector activity leading to plant resistance.

SAR signaling requires increase in plant phytohormone Salicylic acid (SA) and redox changes in positive regulatory protein NONEXPRESSER OF PR GENES1 (NPR1). Increase in
SA causes an important redox reduction in two of the ten conserved cysteines of NPR1, C82 and C216. This also results in a change in state of the oligomerized form of NPR1, which is held together by disulfide bridges, to an active monomeric form which is translocated into the nucleus where it regulates the expression of *PATHOGENESIS RELATED (PR)* genes (Figure 1.3) (Clarke et al., 1998; Fobert and Després, 2005). NPR1 and two of its paralogues NPR1-LIKE PROTEIN 3 (NPR3) and NPR1-LIKE PROTEIN 4 (NPR4) have been discovered to be SA receptors; NPR3 and NPR4 also act as CUL3 adaptors for NPR1 degradation (Fu et al., 2012; Wu et al., 2012).

![Diagram showing the monomerization of NPR1](image)

**Figure 1.3: NPR1 monomerization is essential for SAR.** Increase in SA leads to monomerization and subsequent nuclear translocation of NPR1 leading to transcription of pathogenesis related genes which are required for SAR.

Other phytohormones besides SA are known to influence plant defense response to different pathogens. Crosstalk between SA and some of these phytohormones have been
extensively studied. Jasmonic acid (JA) is a linolenic acid derived phytohormone that is induced during defense against herbivores and necrotrophic pathogens, and plays an important role in defense against these invaders. Most studies on SA and JA crosstalk have shown that both pathways are mutually antagonistic; exogenous treatment with either hormone results in transcriptional reprogramming events that inhibit the pathway related to the other hormone (Gupta et al., 2000; Glazebrook et al., 2003; Koornneef and Pieterse, 2008). Synergistic activity between SA and JA has also been reported (Derksen et al., 2013). Simultaneous treatment of plants with JA and SA results in an NPR1-dependent suppression of JA-responsive gene expression (Figure 1.4) (Spoel et al., 2003).

Ethylene is another phytohormone implicated in plant defense. Ethylene is a hormone most commonly known for its role in ripening, however its effect can be found at all states of plant growth and development. Ethylene plays a role in plant defense by acting synergistically with JA and antagonistically against SA (Schenk et al., 2000; Broekaert et al., 2006).

**Figure 1.4: Phytohormone crosstalk:** SA and JA are mutually antagonistic; ethylene acts synergistically with JA
To determine what proteins are involved in early defense signaling before major transcriptional reprogramming events, we treated wildtype (WT) and NPR1 mutant (npr1-1) Arabidopsis thaliana Columbia (Col-0) ecotype plants with benzo-(1,2,3)-thiadiazole-7-carbothioic S-methyl ester (BTH), a potent analog of salicylic acid not degraded by salicylate hydroxylase (Shah et al., 1999). npr1-1 is an NPR1 ethylmethane sulfonate mutant that shows a small amount of PR gene expression and is unable to respond to various SAR-inducing treatments; they also show increased susceptibility to pathogen infections (Bowling et al., 1994). We determined changes in the protein abundance in WT and npr1-1, and changes in phosphorylation in WT plants after 5 minutes of BTH treatment. Our results include some proteins previously implicated in hormone signaling, and biotic and abiotic stress. To determine the defense related phenotypes of these proteins, we subjected the T-DNA knockout lines of 8 selected proteins to Hyaloperanospora arabidopsidis (Hpa Emwa1, Hpa Emco5 and Hpa Noco2 isolates), Pseudomonas syringae pv. tomato DC3000, and Botrytis cinerea infection. Our results reveal the novel roles of 1 protein in plant defense against p. syringae, 5 proteins in defense against H.arabidopsidis and 3 proteins in defense against B. cinerea.
Results

Mass spectrometric analysis of total proteome and phosphoproteome following BTH treatment reveal changes during early defense signaling

To indentify proteins that are involved in early signaling in plant immunity, above ground tissue from 5 minutes BTH treated wildtype plants were cut and frozen with liquid nitrogen. Control plants were also collected and frozen. Proteins were extracted from the leaves and subjected to mass spectrometry analysis. Proteins used for phosphoproteome analysis were enriched for post translational modification by binding to cerium oxide.

In the total proteome analysis of WT plants, we identified and measured 4,970 proteins; of these, 20 proteins increased in abundance while 28 proteins decreased in abundance after 5 minutes of BTH treatment (Table 1.1, Appendix Table 1). We considered changes in protein abundance or phosphorylation to be significant if the changes were greater than 1.5 fold with a p-value of ≤0.1.

We analyzed the observed proteins using TAIR (The Arabidopsis information resource) and agriGO (GO Analysis Toolkit and Database for Agricultural Community) web based gene ontology tools and database. TAIR GO analysis revealed that 16 of the 49 observed proteins function in response to stress and/or abiotic and biotic stimuli (Table 1.1). AgriGO GO analysis of the biological processes of proteins that change in abundance in comparison to total proteins observed revealed that proteins involved in fatty acid metabolic process was significantly upregulated (p. value of 0.0013, FDR of 0.046) after 5 mins of BTH treatment (Figure 1.5).

From the WT phosphoproteome analysis, we observed 2,384 phosphopeptides of which 47 of these changed in phosphorylation; 19 phosphopeptides were observed to increase in phosphorylation while 28 decreased in phosphorylation following 5 min BTH treatment (Table 1.1, Appendix Table 2). We observed that 12 of the phospho-regulated proteins were annotated by TAIR to be involved in stress response, and/or biotic and abiotic stimuli (Table 1.1). AgriGO
GO analysis of the biological processes of proteins that changed in phosphorylation in comparison to the total phosphoproteins observed did not show any significant GO term enrichment (Appendix Figure 1).

Table 1.1: Summary of mass spectrometry results

Results are based on three biological replicates *significant changes were greater than 1.5 fold with a p-value of ≤0.1

<table>
<thead>
<tr>
<th></th>
<th>Total Proteome</th>
<th>Phosphoproteome</th>
<th>npr1-1 proteome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of proteins measured</td>
<td>4970</td>
<td>1,105 phosphoproteins</td>
<td>1,942</td>
</tr>
<tr>
<td>Changes after BTH treatment*</td>
<td>20 increased</td>
<td>19 phosphorylated</td>
<td>15 increased</td>
</tr>
<tr>
<td></td>
<td>28 decreased</td>
<td>28 dephosphorylated</td>
<td>28 decreased</td>
</tr>
<tr>
<td>Response to stress, biotic or abiotic stimuli</td>
<td>16</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Selected for further studies</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

NPR1 is an SA binding protein with important positive regulatory role in SA-mediated systemic acquired resistance (Cao et al., 1994; Delaney et al., 1995; Wu et al., 2012). To determine the effect of mutations in NPR1 on early changes in protein abundance during plant defense, we treated NPR1 mutant (npr1-1) and wildtype A. thaliana plants with BTH for 5 mins. Above ground tissues were cut, flash frozen and crushed, and the protein was extracted for mass spectrometry analysis. Control WT and npr1-1 were also treated and analyzed in a similar manner.
Analysis of the changes in protein abundance in npr1-1 mutants compared with changes in WT plants after 5 mins BTH treatment led to the identification and measurement 1,942 proteins of which 15 increased in abundance, and 28 decreased in abundance (Table 1.1, Appendix Table 3). TAIR GO annotation revealed that 14 of the observed proteins function in stress and/or abiotic and biotic stimuli (Table 1.1). AgriGO GO annotation of the biological processes of the changes observed did not reveal any significantly upregulated or downregulated GO terms compared to the total proteins observed (Appendix Figure 2).

**Figure 1.5: Go annotation of the biological processes of the total proteome.** Black bars indicate GO annotation of proteins that change in abundance after BTH treatment. Grey bars indicate GO annotation of the total proteins observed. * P<0.05 ** P<0.01 *** P<0.001 **** P<0.0001
Discovering the biological role of selected proteins in defense against biotrophic and necrotrophic pathogens

To determine the biological role of the observed proteins in defense, we selected 4 proteins from the WT total proteome data and 4 proteins from the phosphoproteome data for further studies. The proteins were selected based on bioinformatics and availability of T-DNA knockout lines. T-DNA knockout lines of a total of 8 proteins were obtained (Appendix Table 4) from ABRC and tested for their role in defense against *Hyaloperonospora arabidopsis* (Hpa Emwa1, Hpa Emco5 and Hpa Noco2 isolates), *Pseudomonas syringae* pv. DC3000, and *Botrytis cinerea grape isolate*. The following knockout lines were tested for their role in defense: at1g01610, at1g01960, at1g14250, at2g04880, at3g56370, at4g26530, at5g16990, and at5g54270; their phenotypes were determined based on reproducibility in at least three experiments.

*H. arabidopsis* infection reveals the novel roles of AT1G14250, AT2G04880, AT3G56370, AT4G26530, and AT5G16990 in defense against oomycetes.

Oomycetes are a diverse group of filamentous organisms that have destructive effects on plant growth. Oomycetes *Hyaloperonospora arabidopsis* (Hpa) is an obligate biotrophic pathogen of Arabidopsis. Infection with different isolates of Hpa can reveal the roles of a protein in plant defense including PTI and ETI.

Infection with *Hpa* Emwa1 reveals the role of a protein in PTI and RPP4-mediated ETI against oomycetes. To determine the role of AT1G01610, AT1G01960, AT1G14250, AT2G04880, AT3G56370, AT4G26530, AT5G16990, and AT5G54270 in defense against Hpa Emwa1, 2-week-old seedlings of their T-DNA knockout mutants (Appendix Table 4) and WT (Col-0) were infected with *Hpa* Emwa1 isolate; growth of sporangiophores were scored 5 days
post infection (dpi). *Arabidopsis thaliana* Wassilewskija ecotype (Ws), which is known to show enhanced disease susceptibility to *Hpa* Emwa1, and *npr1-1* was used as a control for this experiment. Upon infection, we observed that *at2g04880, at3g56370,* and *at5g16990* mutants showed enhanced disease susceptibility (Figure 1.6). T-DNA knockout mutant *at1g01610, at1g01960, at1g14250, at3g56370, at4g26530* and *at5g54270* showed comparable susceptibility to WT. This shows that AT2G04880, AT3G56370, and AT5G16990 are required for PTI and RPP4-mediated ETI against *Hpa* Emwa1. However, whether these three proteins play a role in both PTI and ETI or just one pathway cannot be determined with this data.

Infection with *Hpa* Noco2 reveals the role of proteins in PTI against oomycetes in an SA-independent manner. To determine the role of the mutants in defense against *Hpa* Noco2, 2-week-old seedlings of the mutants and WT were infected with *Hpa* Noco2 isolate and the growth of sporangiophores were scored 4 dpi. Ws, which shows complete resistance to *Hpa* Noco2, was used as a control for this experiment. We observed that upon infection of our 8 mutants and *npr1-1* with *Hpa* Noco2, *at2g04480* showed enhanced disease susceptibility. On the other hand *at1g14250, at4g26530,* and *npr1-1* showed partial resistance to *Hpa* Noco2 (Figure 1.7). All the other five mutants showed comparable susceptibility to WT Col-0. This suggests that AT2G04880 is required for SA-independent PTI against *Hpa* Noco2. The results also show that absence of AT1G14250 or AT4G26530 results in resistance against *Hpa* Noco2, which suggests that these protein levels may be reduced or inactivated during defense signaling to provide resistance.

Infection with *Hpa* Emco5 reveals the role of a protein in SA-mediated PTI. To determine the role of the mutants in defense against *Hpa* Emco5, 2-week-old seedlings of the mutants and WT were infected with *Hpa* Emco5 isolate and the growth of sporangiophores were scored 4 dpi. Knockout mutant *eds16*, which shows enhanced disease susceptibility to *Hpa*
Emco5, was used as a control for this experiment. We observed that infection with Emco5 resulted in enhanced disease susceptibility of \textit{at2g04880} (Figure 1.8). The remaining seven mutants showed comparable susceptibility to WT. This shows that AT2G04880 is required for SA-mediated PTI against \textit{Hpa} Emco5.

\textbf{Figure 1.6:} \textit{Hpa} infection reveals the role of 8 selected proteins in defense against \textit{Hpa Emwa1} isolate. Mutant \textit{npr1-1} and ecotype Ws were infected as controls. Susceptibility to \textit{Hpa Emwa1} was measured based on sporangiospore count 5dpi (mean $\pm$ 2xSE). At least three individual experiments were performed with similar results.
Figure 1.7: Hpa infection reveals the role of 8 selected proteins in defense against Hpa Noco2 isolate. Mutant npr1-1 and ecotype Ws were infected as controls. Susceptibility to Hpa Noco2 was measured based on sporangiospore count 4dpi (mean ± 2xSE). At least three individual experiments were performed with similar results.

Figure 1.8: Hpa infection reveals the role of 8 selected proteins in defense against Hpa Emco5 isolate. Mutant npr1-1 and eds16 were also infected as controls. Susceptibility to Hpa Emco5 was measured based on sporangiospore count 4dpi (mean ± 2xSE). At least three individual experiments were performed with similar results.
WRKY1 is required for defense against *Pseudomonas syringae* DC3000

To reveal the role of the 8 selected proteins in PTI against DC3000, 4-5 wks old WT and T-DNA knockout plants were subjected to syringe inoculation with *Pto* DC3000 and bacteria growth was scored 0 and 4 days post infection. The role of the proteins in induced resistance was also assayed. Four to five wks old plants were primed by spraying with 300uM BTH, 3 days prior to infection with DC3000. Bacteria growth was scored 0 and 4 dpi. The mutant npr1-1, which shows enhanced disease susceptibility to *Pto* DC3000, and impaired induced resistance when primed with BTH was used as a control. *at1g01610, at1g01960, at1g4250, at3g56370, at4g26530, at5g16990 and at5g54270* showed bacteria growth similar to WT with or without BTH priming when infected with *Pto* DC3000 (data not shown). The mutant *at2g04880 (wrky1)* showed enhanced disease susceptibility without priming indicating that WRKY1 is necessary for defense against *Pto* DC3000. Priming with BTH resulted in BTH induced resistance relatively comparable to WT revealing that WRKY1 is not necessary for BTH induced resistance (Figure 1.9).

**Infection with *Botrytis cinerea* reveals the novel roles of AT1G14250, AT4G26530 and AT5G54270 in defense against necrotrophic pathogens**

*Botrytis cinerea* is a necrotrophic fungus that can penetrate wounds or natural openings in plants, creating a grey mold phenotype upon infection. *B. cinerea* secretes exopolysaccharides which suppresses the JA pathway by eliciting the SA pathway thus taking advantage of the SA-JA antagonism to infect plants (El Oirdi et al., 2011). To determine the role of the 8 selected proteins in defense against *B. cinerea*, we subjected leaves of 4-5wk old plants to infection with *B. cinerea* grape isolate. The diameters of the lesions were measured in infected leaves four days post infection. *PROTEASOME ALPHA SUBUNIT D2* knockout mutants (*pad2*), known for their deficiency in camalexin biosynthesis and enhanced disease susceptibility to botrytis, was used as
control for this experiment (Ferrari et al., 2003). *B. cinerea* infection revealed that *at1g14250*, *at4g26530* and *at5g54270* have partial but significant resistance to *B. cinerea* compared to WT (Figure 1.10). This shows that absence of AT1G14250, AT4G26530, and AT5G54270 results in resistance to botrytis.

**Figure 1.9: Knockout mutant wrky1 displays enhanced disease susceptibility phenotype.** Mutant plants were syringe inoculated with *Pto* DC3000 (OD$_{600}$=0.002) and scored 0 and 4 dpi. Bars with asterisks were significantly different according to Student’s t test at * P<0.05 ** P<0.01 *** P<0.001 **** P<0.0001
Figure 1.10: Response of selected proteins to B. cinerea. Graphical representation of disease symptoms (diameter of lesions) at 4 d after infection (mean ± SE). At least 16 leaves were inoculated per genotype. At least three independent experiments were performed that yielded similar results. Bars with asterisks were significantly different according to Student’s t test at * P<0.05 ** P<0.01 *** P<0.001 **** P<0.0001
STRING analysis of the protein changes in total proteome, phosphoproteome and npr1-1 reveal potential protein networks and possible mechanisms of action.

To determine the relationship between the changed proteins in our three data sets (total proteome, phosphoproteome and npr1-1 mutant proteome), we combined the list of proteome protein changes, totaling 137 proteins, and performed a STRING analysis of the protein-protein interactions. STRING is an online database of known and predicted protein interactions. It creates interaction based on information from four sources: genomic context, high throughput experiments, co-expression data and prior knowledge from databases. The networks created, consists of protein nodes connected by single or multiple colored edges which represent, connection based on neighborhood, co-occurrence, co-expression, experimental data, database information, text-mining and/or homology (http://string-db.org/). String analyzed 129 of the 137 input proteins and created 13 networks. Most of the proteins were predicted as unattached nodes (Figure 1.1). Further analysis of the networks created by STRING will be discussed in the discussion section of this dissertation.
Figure 1.11: STRING output of interaction between proteins from the total proteome, phosphoproteome and npr1-I proteome analysis. Node size and edge length do not provide any information on the proteins interactions. (Modified from SPRING derived networks)
Discussion

We determined the proteins involved in early defense signaling in WT and npr1-1 plants by analyzing protein extracts from 5mins BTH treated plants using mass spectrometry. We determined changes in the protein abundance in WT and npr1-1, and changes in phosphorylation in WT plants after 5 minutes of BTH treatment. We observed 48 proteins change in abundance, and 47 proteins change in phosphorylation in WT plants. We also observed 43 proteins change in abundance in npr1-1 mutant plants compared to WT. Of the 137 changed proteins that we observed, we selected 4 proteins with changes in abundance and 4 phospho-regulated proteins to determine their biological role in defense against Hyaloperonospora arabidopsidis (Hpa Emwa1, Hpa Emco5 and Hpa Noco2 isolates), Pseudomonas syringae pv. DC3000, and Botrytis cinerea grape isolate.

Changes in cutin biosynthesis proteins in response to BTH treatments

At1g01610 encodes GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 4 (GPAT4), annotated to be involved in lipid biosynthesis. GPAT4 is a bi-functional enzyme with both Sn-2 acyltransferase and phosphatase activity (Yang et al., 2010). In Arabidopsis thaliana, GPAT4 and its homolog PROBABLE GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 8 (GPAT8) are required for the accumulation of cutin monomers (Li et al., 2007). Cutin is the principal constituent of the cuticle, the waxy protective layer covering the epidermis of the leaf. Alterations in cuticular structure have been reported to lead to release of fungi-toxic substances and defense-related changes in gene expression (Chassot et al., 2008).

We observed that GPAT4 decreased in abundance following BTH treatment. Upon infection with Pto DC3000, Hpa, and B. Cinerea, we observed that GPAT4 had a similar
response to pathogen infection as WT suggesting that it is not required for defense against the tested pathogens. However, it is possible that the observed phenotype is related to its redundant function with GPAT8, which may compensate for its function in defense. Yonghua Li et al. observed that the single knockout gpat4 does not show significant differences in cutin accumulation compared to WT, however the gpat4/gpat8 double mutant show significant reduction in C16 and C18 cutin monomer accumulation (Li et al., 2007). In Brassica napus, the gpat4 RNAi line which was designed to silent all the Bn GPAT4 homologs, and decrease overall expression levels by approximately 40% , showed increased cuticle permeability and water loss, and altered cuticle load (Chen et al., 2011). Although we observed reduction in GPAT4, our mass spectrometry results showed no significant changes in homolog GPAT8 following BTH treatment. This suggests that of the two homologs, GPAT4 is targeted for reduction during defense; this may be a signaling activity that turns on other cutin-related defense responses.

STRING analysis assigned GPAT4 to a network of two with CYTOCHROME P450 86A2 (CYP86A2 (AT4G00360)), a cytochrome P450 protein that increased in abundance following BTH treatment (Figure 1.11). The network is based on co-expression (STRING score - 0.462) and co-mention in abstracts (MacGregor et al., 2008; Li-Beisson et al., 2009; Panikashvili et al., 2009; Yeats et al., 2010; Lü et al., 2012). CYP86A2 and members of its subfamily the CYP94B cytochrome P450 monoxygenase proteins, have been implicated in cutin biosynthesis, production of signaling molecules, and prevention of the accumulation of toxic levels of free fatty acids (Pinot et al., 1999; Wellesen et al., 2001; Xiao et al., 2004; Duan and Schuler, 2005). CYP86A2 has also been reported to repress p. syringae type III related genes, and to increase in expression for up to 24hrs after SA treatment, which supports our observation of its increment following 5 mins of BTH treatment. Our results show that a CYP86A2 change in protein abundance occurs very early during defense (Xiao et al., 2004; Rupasinghe et al., 2007)
Further STRING analysis of CYP86A2, GPAT4 and its homolog GPAT8 creates a network of all three proteins where both GPAT homologues connect through CYP86A2 based on co-expression and co-mention in abstracts (Figure 1.12). During defense, GPAT4 protein levels may linked to CYP86A2, in which case while CYP86A2 increases over the course of infection, while GPAT4 reduces during the first 5 mins of infection. The early drop in GPAT4 may trigger other cutin-related defense responses. It is also possible that GPAT4 levels may be biphasic and may change in the direction of abundance after the initial drop in levels. It would be interesting to see the changes in GPAT4 protein levels over longer periods of time, and the effect of defense elicitation on the levels of cutin-related proteins in gpat4, gpat4/gpat8 and cyp86a2 mutants.

**Figure 1.12: STRING network of cutin biosynthesis related proteins GPAT4, CYP86A2 and GPAT8.** CYP86A2 and GPAT4 are connected based on co-expression (black line) and co-mentioned in abstracts (light green line). Upward arrow indicates increase in protein abundance from our total proteome, downward arrow indicates decrease in abundance and NC indicates no change in protein abundance following BTH treatment. Node size and edge length do not provide any information on the proteins interactions. (Modified from SPRING derived networks)
Dephosphorylation of vesicular transport proteins in response to BTH treatment

EMBRYO SAC DEVELOPMENT ARREST 10 (EDA10 (AT1G01960)), another selected protein was observed to be dephosphorylated following BTH treatment while its abundance remained unchanged in our data. We observed that infection with *Hpa Emw1* results in enhanced disease susceptibility phenotype in *at1g01960*, showing that EDA10 (AT1G01960) functions in PTI and RPP4-mediated ETI against oomycetes.

EDA10 is a SEC7 domain containing adenosine diphosphate (ADP) ribosylation factor (ARF) guanine nucleotide exchange factor (GEF) also referred to as BREFELDIN A-INHIBITED GUANINE NUCLEOTIDE-EXCHANGE PROTEIN 3. ARF-GEFs play a functional role in vesicle formation and are inhibited by brefeldin A (Peyroche et al., 1999; Donaldson and Jackson, 2000; Robineau et al., 2000; Raikhel, 2003). Although not much previous knowledge on EDA10’s function exists, EDA10 bears 38% sequence similarity with HOPM INTERACTOR 7 (AtMIN7- AT3G43300), another protein observed in our data to be rapidly dephosphorylated following BTH treatment (Appendix Table 2). AtMIN7 like EDA10 is one of the eight member family of ARF-GEFs proteins and is also referred to as BREFELDIN A-INHIBITED GUANINE NUCLEOTIDE-EXCHANGE PROTEIN 5. AtMIN7 is a target of *P.syringae* HopM1 effector protein. HopM1 interacts with and destabilizes AtMIN7 possibly as a mechanism for inhibiting host vesicle trafficking pathway (Nomura et al., 2006). EDA10 may also function in PTI or ETI defense-related vesicle trafficking against oomycetes.

STRING analysis resulted in a network of two proteins between EDA10 and AT1G21630 based on co-expression (STRING score 0.438); AtMIN7 was not included in this network (Figure 1.13). We observed that like EDA10 and AtMIN7, AT1G21630 were rapidly dephosphorylated following BTH treatment. Although not much regarding the function of AT1G21630 is known, one of its domain is an Eps15 Homology (EH) domain implicated in endocytosis, vesicle
transport and signal transduction (Mayer, 1999); AT1G21630 also has two EF-hand type calcium binding domains (http://harvester.kit.edu/HarvesterPortal). It is interesting to see that these three vesicle transport related proteins became rapidly dephosphorylated following BTH treatment suggesting that dephosphorylation may be an early signal for regulating vesicular transport in plant defense. Phosphomimic and phosphodead mutations in the phosphosites of these proteins may serve to reveal the importance of phospho-regulation in vesicular transport proteins during defense.

Figure 1.13: STRING network of vesicle transport related proteins. EDA10 and AT1G21630 are connected based on co-expression (black line). Downward arrow P indicates decrease in phosphorylation from our phosphoproteome data following BTH treatment. Node size and edge length do not provide any information on the proteins interactions. (Modified from SPRING derived networks)
Changes in NPR1-dependent proteins that are associated with increasing the synthesis of energy and carbon skeleton in response to BTH treatment

AT1G14250 which codes for PROBABLE APYRASE 5 is another protein selected to determine its role in defense. ATP-diphosphohydrolases (apyrase) catalyze the hydrolysis of phosphoanhydride bonds of nucleoside tri and di phosphates in the presence of divalent cations (Handa and Guidotti, 1996). Although not much is known about the function of plant apyrases, Basic Local Alignment Search Tool (BLAST) analysis of APYRASE 5 revealed that it is has strong similarity to ECTONUCLOSIDE TRIPHOSPHATE DIPHOSPHOYDROLASE 1 (ENTPD1) in Rattus norvegicus. Oliveira et al recently reported that ENTPD1 activity is altered on the surface of lymphocytes of rats infected with Trypanosoma evansi, which suggests that ENTPD1 activity may contribute to immune and inflammatory response in infected animals (Oliveira et al., 2012).

We observed that following BTH treatment, APYRASE 5 (AT1G14250) became rapidly reduced in abundance. Infection of T-DNA knockout mutant of APYRASE 5 (at1g14250) with Pto DC3000, Hpa Emwa1, and Hpa Emco5 showed no observable difference in pathogen growth compared to WT. However, Infection with Hpa Noco2 showed at1g14250 mutants to be partially resistant; suggesting that absence of APYRASE 5 enhances SA-independent PTI against Hpa Noco2. Knockout mutant at1g14250 also showed partial disease resistance to necrotroph B. cinerea, suggesting that absence of AT1G14250 not only results in resistance to biotrophic but also necrotrophic pathogens. This suggests that APYRASE 5 may play an important role in SA and JA signaling, and that it can be rapidly reduced to increase resistance to either biotrophs or necrotrophs.

During pathogen infection, plants are faced with an energy-related opportunity cost where processes involving growth and development must be forgone to allocate resources to
defense response. Activation of defense places considerable demand on the area of infection which requires carbon skeletons to synthesize new molecules, and energy to fuel biosynthesis (Bolton, 2009). Apyrases catalyze the hydrolysis of ATP and therefore do not favor the production of energy for defense related purposes (Figure 1.15). In arabidopsis, over-expression of APAYRASE 5 homologs, APYRASE 1 (APY1) and APYRASE 2 (APY2) result in enhanced growth in hypocotyls. On the other hand, apy1apy2 double mutant exhibits stunted growth and increased ATP production (Wu et al., 2007). It is therefore not surprising that BTH treatment induced rapid reduction of APYRASE 5 levels, which may decrease the energy allocated to growth, and result in increased ATP levels that can be diverted to defense against different pathogens. This may also explain the partial resistance to biotrophs and necrotrophs that we observed in at1g14250. It would be interesting to see if there is an increase in ATP production in at1g14250, and if there is increased plant growth in APYRASE 5 overexpression lines.

STRING analysis of our data put AT1G14250 (APYRASE 5), AT3G22960 (PKP-ALPHA), AT4G26530, AT2G01140, AT3G59920 (ATGDI2), and AT3G15570 in the same network (Figure 1.14). In the network, AT1G14250 is connected to PLASTIDIAL PYRUVATE KINASE 1 (PKP-ALPHA) based on curation (STRING score 0.900), and annotated involvement in purine metabolism. PKP-ALPHA is a protein essential for the production of oil in developing seeds (Sangwan et al., 1992; Baud et al., 2007; Lonien and Schwender, 2009). Although there are no known defense related roles of PKP-ALPHA, our data shows PKP-ALPHA to be reduced in abundance in npr1-1 mutants compared with WT following BTH treatment, suggesting that it requires NPR1 for stability and normal levels during defense. NPR1 is an SA receptor and regulator that is required for defense against biotrophs, for SA-induced suppression of JA signaling, and for SAR (Spoel et al., 2003; Wu et al., 2012). PKP-ALPHA may function with NPR1 in any of these processes.
Plant respiration can be used to generate energy and carbon skeleton needed for defense. Respiration can be divided into glycolysis, TCA cycle and mitochondrial electron transport. In glycolysis, glucose is converted to pyruvate resulting in the net gain of ATP. The Pyruvate from the glycolysis pathway can be oxidized to acetyl CoA which enters the TCA cycle to generate ATP and oxidative phosphorylation. Oxidative phosphorylation from the TCA cycle generates reducing equivalents which can be used in the electron transport chain to produce ATP. The oxidative pentose phosphate pathway (OPP) is a pathway involved in the oxidation of glucose-6 phosphate to generate NADPH and 5-carbon sugars; this pathway shares intermediates with glycolysis. These energy generating pathways are relevant to the network currently being discussed because key members of these pathways have been implicated in defense. For example glucose-6-phosphate was reported by Dong et al, to be important for NPR1 localization and to be a key part of SA signaling (Dong, 2004; Bolton, 2009). Furthermore, upregulation of key rate limiting enzymes from the TCA cycle such as citrate synthase and alpha-ketoglutarate dehydrogenase have been observed during defense (Wiegand and Remington, 1986; Bolton, 2009). Three of the six proteins in this STRING network are involved in one of these energy producing pathways.

PKP-ALPHA is a pyruvate kinase that is necessary for the production of pyruvate for the TCA cycle (Figure 1.15). PKP-ALPHA may function upstream of this, it may be important for early NPR1-dependent signaling events that ensure energy production from the TCA cycle for defense. It would be interesting to test *PKP-ALPHA* mutants for possible defense related phenotypes which may be as a result of altered energy production from the TCA cycle.

In the STRING network, PKP-ALPHA is connected to two fructose aldolase proteins AT2G01140 (PROBABLE FRUCTOSE-BISPHOSPHATE ALDOLASE 3) and AT4G26530 (FRUCTOSE-BISPHOSPHATE ALDOLASE 5); both of which are connected to each other in
the network by multiple edges. PKP-ALPHA is connected to both proteins based on database curation and being co-mentioned in abstracts. All three proteins are curated based on KEGG to be involved in metabolic pathways, carbon fixation in photosynthetic organisms, glycolysis and gluconeogenesis, pentose phosphate pathway as well as biosynthesis of secondary metabolites.

In our mass spectrometry data, AT2G01140 (PROBABLE FRUCTOSE-BISPHOSPHATE ALDOLASE 3) is decreased in abundance in npr1-1 mutant background showing that it is NPR1 dependent like PKP-ALPHA. Although AT2G01140’s biological function is unknown, it has been reported to decrease in abundance following H$_2$O$_2$ treatment (Sweetlove et al., 2002).

AT4G26530 (FRUCTOSE-BISPHOSPHATE ALDOLASE 5) is observed in our data to be rapidly dephosphorylated following BTH treatment. We also observed that AT4G26530 knockout mutants showed partial resistance to Hpa Noco2 and B.cinerea like at1g14250, a member of this network discussed earlier, but unlike AT1G14250, AT4G26530 is dephosphorylated in response to BTH. AT4G26530 may also be regulated by changes in SA and JA to favor defense over energy production.

AT4G26530 and AT2G01140 are connected in the network based on curation (mentioned earlier), co-mentioned in abstracts (Kim et al., 2010), co-occurrence in eukaryotes and bacteria, and in the biochemical reactions involved in exocytosis of platelet alpha granule contents (Reactome pathways, www.reactome.org). AT2G01140 (PROBABLE FRUCTOSE-BISPHOSPHATE ALDOLASE 3) and AT4G26530 (FRUCTOSE-BISPHOSPHATE ALDOLASE 5) are the other two glycolysis-related enzymes in this network. Fructose-bisphosphate aldolase functions in the reversible conversion of fructose 1-6 bisphosphate to dihydroxyacetone phosphate and glyceraldehyde -3-phosphate in glycolysis (Figure 1.15). Aldolases also function in the gluconeogenesis pathway and Calvin cycle which are anabolic processes. AT2G01140’s stability is dependent on NPR1, which suggests that it may be needed
for NPR1 dependent regulation of the glycolysis pathway. AT4G26530 however becomes phosphorylated after BTH treatment suggesting that AT4G26530 undergoes BTH dependent phosphorylation to achieve its role in glycolysis which may be to preferentially catalyze processes that favor defense. Our results also show that although both of these aldolases are annotated to function in the same pathway and in the same reactions they defer in their mode of regulation and possibly their function.

PKP-ALPHA is also connected to RAB GDP DISSOCIATION INHIBITOR 2 (ATGDI2, AT3G59920) based on experimental evidence of their physical interaction (Consortium, 2011). Our data shows that like PKP-ALPHA, ATGDI2 is reduced in abundance in npr1-1 mutant background compared to WT following BTH treatment. Although not much is known about ATGDI2’s function, it is known that RAB GDP dissociation inhibitors regulate GDP-GTP exchange reaction of RAB family proteins involved in vesicular trafficking between organelles (Entrez Gene: GDI2 GDP dissociation inhibitor 2). ATGDI2 may function in NPR1-dependent vesicular trafficking necessary for defense signaling.

AT4G26530 is connected to AT3G15570 (NON-PHOTOTROPIC HYPOCOTYL 3(NPH3)) based on co-expression (STRING score - 0.518). NPH3 is a photoreceptor interacting protein that is essential for phototropism. We observed that AT3G15570 is phosphorylated in response to BTH treatment. A well characterized member of the NPH3 family, AT5G64330, has been reported to be phosphorylated in low blue light condition and dephosphorylated in high light conditions (Pedmale 2007). In low light conditions, AT5G64330 which functions as a substrate adapter for CULLIN-RING E3 ubiquitin ligase CRL3\textsuperscript{NPH}, monoubiquinates PHOTOTROPIN 1 (PHOT1), subsequently resulting in its clathrin mediated transport from the plasma membrane to the cytosol (Sullivan et al., 2008; Kaiserli et al., 2009; Roberts et al., 2011). Endocytosis of small amounts of PHOT1 is capable of fundamentally changing intracellular signaling. Roberts et al,
also proposed that PHOT1 transport is coupled with auxin transport (Roberts et al., 2011). Our data shows that phosphorylation of AT3G15570 is BTH induced, suggesting that AT3G15570 is an NPH3 that functions in early defense signaling. Interestingly, we also observed PHOT1, a mediator of blue light induced growth enhancements, to be rapidly dephosphorylated after BTH treatment (Appendix table 2). However its dephosphorylation site is not one of the four previously reported blue light induced phosphosites (Sullivan 2008).

We propose that during defense signaling, AT3G15570 becomes rapidly phosphorylated, and it interact with BTH-induced dephosphorylated PHOT1 to cause monoubiqitination and subsequent clathrin mediated endocytosis of PHOT1. This may result in significant changes to defense related signaling including ones that limit growth. This may also be coupled to the transport of SA. It would be interesting to see the affinity of AT3G15570 for PHOT1 during SA/BTH treatment and to test the susceptibility of phot1 to pathogen infection which may be required for defense due to its role in stomatal opening and possibly in SA signaling. PHOT1 dephosphorylation may also be required to divert energy from plant growth towards defense. It would be interesting to see if phospho-mutants of AT3G15570 and PHOT1 affect plant growth and immunity.

This network shows how the BTH mass spectrometry data predicts possible early mechanisms that ensure the diversion of energy from growth and development to energy for defense and biosynthesis of carbon skeleton. These members may also be essential players in JA-SA signaling. Some of this network’s proteins showed NPR1-dependent roles in defense.
Figure 1.14: STRING network of NPR1-dependent proteins that are associated with increasing the synthesis of energy and carbon skeleton in response to BTH treatment. PKP-ALPHA is connected to AT1G14250 to AT3G59920 based on curation (blue line). PKP-ALPHA is also connected to AT4G26530 and AT2G01140 based on curation and abstract (blue and light green line). AT4G26530 and AT2G01140 are connected based on database curation, co-occurrence, homology, and co-mention in abstracts (light blue, dark blue, violet and light green lines respectively). AT3G15570 connected to AT4G26530 based on coexpression (black line). Upward arrow P indicates increase in phosphorylation and downward arrow P indicates decrease in phosphorylation from our phosphoproteome data. Downward arrow indicates decrease in abundance and downward npr1-1 arrow indicates reduction in abundance in npr1-1 data. Node size and edge length do not provide any information on the proteins interactions. (Modified from SPRING derived networks)
Figure 1.15: Primary metabolism pathway involved in defense  HK, hexokinase; PGI, phosphoglucone isomerase; Ald, Aldolase; PFK, phosphofructokinase; F2PDH, fructose bisphosphatase dehydrogenase; TPI, triose phosphate isomerase; GAPDH, glyceraldehyde phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; EN, enolase; PK, pyruvate kinase; LDH, lactate dehydrogenase; G6DPH, glucose-6-phosphate dehydrogenase; GL, gluconolactonase; 6PGDH, 6-phosphogluconate dehydrogenase; TK, transketolase; CS, citrate synthase; PDH, pyruvate dehydrogenase; AC, Aconitase; IDH, isocitrate dehydrogenase; α-KGDH, α-ketoglutarate; SCS, Succinyl-CoA synthetase; SDH, succinate dehydrogenase; FM, fumarase; MDH, malate dehydrogenase.
**WRKY1 is required for defense against multiple pathogens**

AT2G04880 encodes ZINC DEPENDENT ACTIVATOR PROTEIN 1 (ZAP1) also known as WRKY1. For the purpose of its homology to defense related WRKYs, we shall refer to it as WRKY1 in this dissertation. WRKY1 is a member of the WRKY superfamily of transcription factors. WRKY transcription factors have one or two WRKY domains defined by the conserved WRKYGQK amino acid and zinc finger-like domains. WRKY1 belongs to the group1 WRKY transcription factors which have two functionally distinct WRKY domains.

WRKYs have preferential binding for W boxes which contain TTTGACT conserved sequence, and an invariant core that is essential for WRKY1’s function and binding (Eulgem et al., 2000). Although not much is known about WRKY1’s role in defense, WRKY1’s expression has been shown to be partially dependent on npr1-1.

Blast analysis of WRKY1 shows high sequence similarity with other group1 WRKYs such as WRKY33 (AT2G38470), WRKY3 (AT2G03340) and WRKY4 (AT1G13960). WRKY33 is an important protein that mediates the crosstalk between JA and SA. It is rapidly induced in expression by pathogen attack (Lippok B 2007). It is also required for defense against botrytis (Zheng 2006, Birkenbilirp 2012). WRKY3 and WRKY4 are two closely related WRKY proteins that also have a high sequence similarity with WRKY1. **WRKY3 and WRKY4** expression is induced by avirulent *P. syringae* infection and SA treatments (Dong et al., 2003). It has also been shown that they play a positive role in resistant against necrotrophs, and a negative role in the resistance against biotrophs (Lai et al., 2008).

Our mass spectrometry data shows that upon treatment with BTH, WRKY1 becomes rapidly dephosphorylated at a novel site (DYNSPtAK). WRKY1 abundance seems to increase with BTH treatment although the p-value does not make the specified cutoff for significance (data not shown). Our mass spectrometry result suggests that WRKY1 activity may be regulated by
rapid dephosphorylation upon BTH treatment to trigger defense signaling. *WRKY1* mutants (at2g04880) show enhanced disease susceptibility phenotypes to all strains of *Hpa* tested and to DC3000, and slight susceptibility to *botrytis*. Infection with these various pathogens resulted in susceptibility, indicating that *WRKY1* plays a role in plant defense against a broad range of pathogens. This suggests that unlike some of the other afore mentioned group1 proteins, *WRKY1* plays a positive role in defense against biotrophs and necrotrophs. STRING analysis did not connect *WRKY1* (ZAP1) with any other observed proteins (Figure 1.16).

**Figure 1.16: STRING network of WRKY1.** *WRKY1* was not connected to any other protein on the network. Downward arrow P indicates dephosphorylation. Node size and edge length do not provide any information on the protein interactions. (Modified from SPRING derived networks)

**STRING network of PER63, IRK and AGO4**

AT3G56370 encodes INFLORESENCE AND ROOT APICES RECEPTOR LIKE KINASE (IRK). We observed IRK to increase in abundance following 5 mins BTH treatment (Hattan et al., 2004). IRK is a member of the dominant class of receptor-like proteins with leucine rich repeats (LRR). The LRR motifs generally function to provide protein-protein interaction in
various processes including hormone signaling. IRK contains a leucine rich repeat domain, a transmembrane domain, and a serine kinase domain. Xa21 and BRI1 are two IRK homologs that play a role in plant defense (Song et al., 1995; Kemmerling et al., 2007). BRI1, was observed in our phosphoproteome data to be rapidly phosphorylated following BTH treatment. This suggests that the defense related phosphorylation of BRI1 happens within minutes of pathogen infection (Appendix Table 2).

We observed that upon infection of T-DNA knockout at3g56370 (irk) with DC3000, at3g56370 showed similar susceptibility to WT, suggesting that IRK is not required for defense against P. syringae. Infection with Hpa Emw1 showed enhanced disease susceptibility in at3g56370, showing that IRK is required for defense against Hpa Emw1 and therefore required for PTI and RPP4-mediated ETI. In defense response to Hpa Noco2, Hpa Emco5, and botrytis, at3g56370 showed comparable susceptibility to WT. IRK may be one of the LRR receptor proteins that mediate early defense signaling and is required for defense against pathogen infection. Although not much regarding its kinase activity is revealed in our data, it would be interesting to see if its published kinase activity has a defense related function.

STRING analysis assigned IRK to a network of three proteins, where IRK serves as the node connecting AGO4 (AT2G27040) and PER63 (AT5G40150) based on co-expression (STRING score 0.609, score 0.408 respectively) (Figure 1.17). In our mass spectrometry data, AGO4 and PER63 were observed to decrease in abundance following 5 mins BTH treatment.

ARGONAUTE 4 (AGO4) is a PAZ/PIWI domain containing protein involved in RNA-directed DNA methylation (Cerutti et al., 2000; Zilberman et al., 2003; Zilberman et al., 2004). AGO4 is required for resistance to virulent (Pto. DC3000), avirulent (Pto. DC3000 avrRpm1) and nonhost Pseudomonas syringae (P.s. tabaci) (Agorio and Vera, 2007).
AT5G40150 encodes PEROXIDASE 63 (PER63), a peroxidase family protein. Peroxidases are haem containing enzymes that use hydrogen peroxide as an electron acceptor in catalyzing oxidative reactions (Nelson 1994). Peroxidases generate about half the H₂O₂ accumulated in response to microbe-associated molecular patterns (MAMPs) treatment (O’Brien et al., 2012). PER63 bears 37% similarity to PEROXIDASE CB (PRXCB (AT3G49120)), another protein observed in our data set to reduce in abundance following BTH treatment (Appendix Table 1). PRXCB was assigned as a disconnected node by STRING (Figure 1.17).

Figure 1.17: STRING network of PER63, IRK and AGO4. IRK served as the node connecting AGO4 (AT2G27040) based on co-expression and PER63 based on co-expression (black line). PRXCB is unconnected. Downward arrow indicates reduction in abundance, while upward arrow indicates increase in abundance. Node size and edge length do not provide any information on the protein interactions. (Modified from SPRING derived networks)
Oxidative stress response proteins reduces in abundance

AT5G16990 codes for NADP-DEPENDENT ALKENAL DOUBLE BOND REDUCTASE P2 also known as P2 (Babiychu 1995, Mano 2002). P2 was observed to decrease in abundance following BTH treatment. P2 is a member of the NADP dependent alkenal double bond reductase which has been implicated in the detoxification of alpha and beta unsaturated aldehydes, and ketones, therefore serving as a universal detoxifier of carbonyls (Dick et al., 2001; Mano et al., 2005). P2 has been observed to confer tolerance to oxidative stress in yeast against diamide, a thiol oxidizing drug (Babiychuk et al., 1995). We observed that knockout mutant at5g16990 (Salk_052140) showed comparable phenotype to WT when infected with DC3000, Hpa Noco2, Hpa Emco5 and botrytis. However infection with Hpa Emwa1 resulted in enhanced disease susceptibility. P2 might function in early signaling involving mass oxidative stress response necessary for PTI and RPP4-mediated ETI. STRING online database did not connect AT5G16990 (P2) with any other observed proteins (Figure 1.18).

Figure 1.18: STRING network of NADP-DEPENDENT ALKENAL DOUBLE BOND REDUCTASE P2 AT5G16990 was not connected to any other protein on the network. Downward arrow indicates decrease in abundance. Node size and edge length do not provide any information on the protein interactions. (Modified from SPRING derived networks)
Chloroplast-related protein network show NPR1 dependency

AT5G54270 codes for LIGHT HARVESTING CHLOROPHYLL B-BINDING protein 3 (LHCB3). LHCB3 is a member of the light harvesting chlorophyll a/b protein complex (LHCs) super gene family, which are antenna pigments that mediate light interception towards the reaction center (Jansson 1999, Jakowski 2001). There are three LHCII proteins associated with photosystem II (PSII), LHCB1, LHCB2 and LHCB3. Unlike LHCB1 and LHCB2, LHCB3 does not contain N-terminal phosphorylation site that is involved in state transition.

We observed phosphorylation of LHCB3 in a novel site following BTH treatment (Appendix Table 2). When LHCB3 knockout mutant (at5g54270) was subjected to DC3000 infection, we observed comparable susceptibility to WT. Infection with all isolates of Hpa also showed comparable susceptibility to WT. However, infection with Botrytis cinerea resulted in partial enhanced disease resistance suggesting that LHCB3 may function in the SA related JA suppression; therefore knockout of LHCB3 relieves JA suppression resulting in resistance to botrytis. Our data also suggests that although LHCB3 is the only LHCII trimer that does not contain a phosphorylation site involved in state transition, its phosphorylation may serve the purpose of early defense signaling (Jenny et al., 2003). Interestingly, interacting protein of LHCB3, LHCB2.1 was observed to decrease in abundance in npr1-1 mutants (Appendix Table 3). Danker et al reported that LHCB3 knockout mutants compensate for the lack of LHCB3 by producing increasing amount of LHCB1 and LHCB2 (Damkjær et al., 2009). It is possible, that during early defense, LHCB3’s role in defense is activated by SA-dependent phosphorylation, which allows it to maintain its suppression on JA pathway proteins. This phosphorylation may be JA-dependent. LHCB3’s phosphorylation may also be related to the NPR1-dependent stability of LHCB2.1 which in turn favors SA signaling in SA-JA crosstalk. On the other hand, the absence of functional NPR1 results in instability of LHCB2.1, and possibly a non-phosphorylated LHCB3.
during defense, which favors the JA pathway. This is partially supported by knockout mutants of LHBC3 which are partially resistant to botrytis. This hypothesis can be tested by determining the phosphorylation state of LHBC3 in npr1-1, which should remain unchanged after BTH treatment if it is dependent on NPR1.

STRING created a network of 6 proteins, LHCB3, LHCB2.1, AT1G52220, CXIP1 (CAX INTERACTING PROTEIN 1, AT3G54900), AT3G56010 and RAT5 (RESISTANT TO AGROBACTERIUM TRANSFORMATION 5, AT5G54640) (Figure 17). AT1G52220 was connected to CXIP1, LHCB3 and AT3G56010 based on co-expression (STRING score - 0.429, 0.843, and 0.646 respectively). CXIP1 and LHCB3 were connected based on co-expression (STRING score 0.419) and LHCB3 was connected to LHCB2.1 based on co-expression (STRING score 0.545), co-mention in abstracts, and high sequence similarity. RAT5 was connected to AT1G52220 based on co-mention in abstracts; however based on personal curation, the connection in the abstract does seem strong enough to justify an interaction between both proteins (Krause 2012). CXIP1, AT1G52220, AT3G56010, LHCB2.1 and RAT5 were observed to be reduced in abundance in npr1-1 mutant proteome data, suggesting that the stability of these proteins during early defense is dependent on NPR1. This may also function in the SA-induced suppression of the JA pathway during infection by a biotroph, which is supported by the partial resistance to botrytis observed in LHCB3 mutants, and that have been previously observed in knockout mutants of CXIP1 homologs such as grx480, and roxy18 (Ndamukong et al., 2007), Ndamukong 2011).

AT1G52220 and AT3G56010 are chloroplast located transmembrane proteins with unknown function (Kleffmann et al., 2004; Peltier et al., 2004). AT1G52220 bears 49% sequence similarity with THYLAKOID MEMBRANE PHOSPHOPROTEIN OF 14 KDA (TMP14), a transmembrane protein that is usually phosphorylated and located in photosystem I (TAIR
BLAST). CXIP1 is a chloroplast localized glutaredoxin involved in homeostasis, with possible roles in protecting cells from oxidative damage (Cheng et al., 2006). This network consists of chloroplast proteins that, which with the exception of LHCB3, are observed to be NPR1 dependent, requiring NPR1 for stability. This is interesting as it shows the dependency of defense-related chloroplast proteins on NPR1.

Figure 1.19: STRING network of NPR1-dependent chloroplast proteins. AT1G52220 was connected to CXIP1, LHCB3 and AT3G56010 based on co-expression (black line). CXIP1 and LHCB3 were connected based on co-expression (black line) and LHCB3 was connected to LHCB2.1 based on co-expression, co-mention in abstracts (light green), and high sequence similarity (grayish blue). RAT5 was connected to AT1G52220 based in co-mention in abstracts (light green)Upward arrow P indicates increase in phosphorylation from our phosphoproteome data. Downward npr1-1 arrow indicates reduction in abundance in npr1-1 data. Node size and edge length do not provide any information on the protein interactions. (Modified from SPRING data output)
Conclusion

The data in this chapter shows that of the 8 selected proteins from our mass spectrometry data, 1 protein is implicated in defense against DC3000, 5 are implicated in defense against one or more strains of Hpa, and 3 proteins are implicated in defense against botrytis. Our mass spectrometry results and our bioassay results were combined to create and explain defense related networks that are involved in cutin biosynthesis, vesicular transport, generation of energy and carbon skeleton biosynthesis, and NPR1-dependent SA-JA signaling. These networks show how mass spectrometric data and bioinformatic tools based on known experimental evidence and predicted protein relationships can be used in combination with our infection bioassay results to explain the biological function of different proteins in defense.
CHAPTER 2

RELATIVE OF EARLY FLOWERING 6 (REF6) PLAYS COMPLEX ROLES IN PLANT IMMUNITY

Introduction

Plants are exposed to beneficial and detrimental environmental factors that affect their growth and development on a regular basis. Plants have sophisticated regulatory mechanisms that allow them to efficiently adapt to stress. Epigenetic modification is one way that plants control stress adaptation. Epigenetic modifications are changes in gene expression or cellular phenotype by mechanisms other than changes in the underlying DNA sequence (Berger et al., 2009). Epigenetic modification includes DNA methylation and post translational modification of histones such as acetylation, deacetylation, methylation, demethylation, phosphorylation, ubiquitination, biotinylation and sumoylation (Chen et al., 2011).

Histone methylation is an addition of one, two or three methyl groups to the lysine or arginine of the N-terminal tail of a histone; a process carried out by histone methyltransferases (Kouzarides, 2007). These modifications play important roles in genome stability, chromatin structure, and developmental reprogramming and transition. Histone di-methylation of H3K9 and tri-methylation of H3K27 are often linked with negative gene regulation while tri-methylation of H3K4 and H3K36 are linked with gene expression (Bedford and Clarke, 2009; Mosammaparast and Shi, 2010; Chen et al., 2011). Histone methylation was once thought of as irreversible modifications; however, it is now well known that histone methylation can be removed by transcription regulators known as histone demethylases.
We are particularly interested in RELATIVE OF EARLY FLOWERING 6 (REF6) a histone demethylase previously reported to function in the demethylation of H3K27me3 (Lu et al., 2011). In chapter 1, the results from our BTH treated WT and npr1-1 proteomic analysis was discussed. We observed that following 5 mins BTH treatment, REF6 became rapidly phosphorylated in two novel sites that have not been previously reported, serine 683 and serine 1092. Although, the p-value of the observed phosphorylation events, are above the cutoff stipulated in chapter 1, we decided to further the study this protein because of its previously reported diverse roles in hormone signaling (brassinosteroids), positive regulation of the flowering, and its diverse knockout phenotypes.

REF6 is one of two Jumonji N/C domain containing proteins that interact with BRI1-EMS-SUPPRESSOR 1(BES1), the other protein being close homolog EARLY FLOWERING 6 (ELF6). BES1 is a transcription factor involved in brassinosteroid response (Yu et al., 2008). REF6 knockout mutants have increased expression of FLOWERING LOCUS C (FLC) and thus exhibit a late flowering phenotype (Noh et al., 2004). They also have shorter petioles and leaf blades, ectopic and increased H3K27me3 level, and decreased mRNA expression of hundreds of genes involved in regulating developmental patterning and responses to various stimuli (Lu et al., 2011). Constitutive over-expression of REF6 leads to very small plants with small petioles and leaf blades. This suggests that REF6 levels are tightly regulated; decrease or increase of REF6 beyond certain levels produces observable growth phenotypes.

To determine the role of REF6 in defense, we subjected REF6 knockout mutants and overexpression lines to infection with various pathogens. We show that ref6 mutants exhibit enhanced disease susceptibility to Pseudomonas syringae pv. tomato DC3000. We also show that ref6 mutant plants primed with BTH prior to infection with Pto DC3000 show enhanced diseases resistance. Furthermore in terms of PTI and ETI we show that REF6 is necessary for full flg22
response suggesting possible roles in PTI, however REF6 is not necessary for RPM1 signaling. These finding suggests that REF6 plays a complex role in plant immunity.

Results

Mass spectrometric analysis reveals REF6 to be rapidly phosphorylated following 5 minutes BTH treatment

To identify proteins that are involved in early signaling in plant immunity, *Arabidopsis thaliana* plants were treated with BTH for 5 minutes, and the plants were cut and frozen with liquid nitrogen. The protein was extracted from these plants and subjected to mass spectrometric analysis. The detail of the plant treatments, analysis, and results is discussed in Chapter 1. We observed that following 5 mins BTH treatment, REF6 became phosphorylated in two sites that have not been previously reported, serine 683 and serine 1092. Although the p-value of the observed phosphorylation events are above the cutoff stipulated in chapter 1 (p-value < 0.1), we decided to further the study of this protein because of its function as a histone demethylase and its known role in hormone signaling.

**REF6** T-DNA insertion mutants show enhanced disease susceptibility and enhanced disease resistance to *Pto DC3000*.

To determine the role of REF6 in plant defense against pathogen infection, **REF6** mutant *ref6-1* (salk_001018C) was subjected to priming with silwet (control) or BTH, and infected with *Pto DC3000* 3 days post priming. Knockout mutants *ref6-1* primed with silwet (control) showed higher bacteria growth than WT thus showing enhanced disease susceptibility to *Pto DC3000* (Figure 2.1). Upon priming with BTH, *ref6-1* plants showed lower bacteria growth than in WT plants primed with BTH, showing that *ref6* plants undergo enhanced disease resistance when treated with BTH (Figure 2.1).
Figure 2.1: REF6 is required for disease resistance. ref6-1 plants primed with silwet (blue bar) or 300uM BTH (red bar) were infected with Pto DC3000 (OD
t=0.0002) and scored 4 days post infection (dpi). * P<0.05 ** P<0.001 *** P<0.0001

Ref6 is required for RPP4-mediated ETI and PTI against Hpa Emwa1

To determined if REF6 defense role can be observed in response to other pathogens, knockout REF6 lines ref6-1 (salk_001018) and ref6-2 (salk_122006) were infected with the following isolates of Hyaloperonospora: Hpa Emwa1, Hpa Noco2, and Hpa Emco5. Differences between these strains are elaborated on in chapter 1. Infection bioassay revealed that REF6 is required for defense against Hpa Emwa1 but not Hpa Emco5 or Hpa Noco2. Since infection with Emwa1 reveals the role in RPP4 and PTI, this data shows that REF6 is necessary for RPP4-mediated ETI and PTI against Hpa Emwa1 (Figure 2.2A, 2.2B and 2.2C).
Figure 2.2: REF6 is required for defense against *Hpa* Emwa1 isolate. Ref6-1 was infected with (A) *Hpa* Emwa1 isolate, (B) *Hpa* Emco5 isolate and (C) *Hpa* Noco2 isolate. *npr1-1*, eds16 and Ws were also infected as controls. Susceptibility to *Hpa* was measured based on sporangiospore count 4 or 5dpi (mean ± 2xSE). At least three individual experiments were performed with similar results. * P<0.05 ** P<0.001 *** P<0.0001 **** P<0.0001

**REF6 is not required for RPM1 signaling**

Due to our observation of REF6’s possible role in RPP4-mediated ETI, and the observation that REF6 is reduced in phosphorylation during RPM1-mediated ETI (unpublished data), we decided to determine if REF6 is required for RPM1-mediated defense against effector protein avrRpm1. Mutants of *REF6* and WT plants were infected with *Pto* AvrRpm1 and compared to plants infected with *Pto* DC3000. The presence of RPM1 in Col-0 plants results in
incompatible interactions which results in lower bacteria growth in WT plants infected with Pto AvrRpm1 than in those infected with Pto DC3000. We observed that the bacteria growth of both WT plants and ref6 mutant plants was lower 4 days post infection with Pto AvrRpm1 than in plants infected with Pto DC3000 (Figure 2.3). This suggests that ref6 mutant plants can undergo compatible interaction in the presence of avrRpm1 effectors and that REF6 is not required for RPM1-mediated defense against Pto AvrRpm1.

![Figure 2.3: REF6 is not required for RPM1 signaling.](image)

**Figure 2.3: REF6 is not required for RPM1 signaling.** (A) Pto DC3000 (OD$_{600}$=0.0002) infection (B) Pto AvrRpm1 infection (OD$_{600}$=0.0002) bacteria growth is scored 4 days post infection

**REF6 is required for full flg22 response**

In brassinosteroid (BR) signaling, brassinolide binds to BRASSINOSTEROID INSENSITIVE 1 (BRI1) receptor resulting in its heterodimerization and transphosphorylation with BRI1-ASSOCIATED KINASE 1 (BAK1). This results in an activated form of BRI1 which phosphorylates downstream BR signaling kinases leading to downstream events including the phosphorylation of BRI1 SUPPRESSOR 1 (BSU). BSU dephosphorylates and inactivates BRASSINOSTEROID INSENSITIVE 2 (BIN2), leading to nuclear accumulation of two
transcription factors BRASSINAZOLE-RESISTANT 1 (BRZ1) and BRI1-EMS-SUPPRESSOR 1 (BES1). BRZ1 and BES1 are important for the regulation of BR responsive genes. REF6 has been shown to bind to BES1 (Yu et al., 2008; Lin et al., 2013). During plant immunity the PAMP flagellin binds to FLAGELLIN-SENSITIVE 2 (FLS2) resulting in the transphosphorylation of BAK1. BOTRYTIS-INDUCED KINASE 1 (BIK1) associates with this complex and is phosphorylated by BAK1. BIK1 is known to positively regulate plant immunity. Mutations in BIK1 results in impaired flg22 mediated induced resistance (Lin et al., 2013).

Given our observed REF6 role in defense and its known interaction with BES1, we aimed to determine if REF6 plays a role in flg22-mediated induced resistance. Knockout mutant ref6-1 plants and WT plants were primed with flg22, a synthetic peptide that corresponds to a highly conserved part of the flagellin N terminus, and acts as a potent defense elicitor. REF6 mutants and WT plants were primed with flg22 24hrs prior to infection with Pto DC3000. We observed that WT plants primed with flg22 showed reduced bacteria growth in comparison to unprimed WT plants showing the ability of flg22 to induce resistance against further infection (Figure 2.4). In ref6-1 plants, although there is a reduction in bacteria growth compared to the control primed ref6-1 plants, there is higher bacteria growth in ref6 mutants primed with flg22 than WT primed with flg22. This result suggests that REF6 is necessary for full response to flg22.

**Inducible overexpression of REF6**

The interesting roles of REF6 in defense against different pathogen led us to investigate REF6 overexpression lines including WT, phosphomimic mutants, phosphodead mutants and deletion of zinc finger binding domain mutant. It was reported by Lu et al, that constitutive overexpression of REF6 results in stunted transgenic plants with small leaves (Figure 2.5). Due to the difficulty that we anticipated in the handling of these plants, we cloned REF6 WT genomic DNA, phospho -mutants (S683E, S683D, S683A, S1092E, S1092D, S1092A) and zinc finger
domain deletion mutant named **REF6FRAG7** into a glucocorticoid inducible system vector (Figure 2.5 and 2.6).

![Figure 2.4: REF6 is required for full response to flg22. ref6-1 plants primed with 1uM flg22 and control plants were injected with Pto DC3000 (OD_{600}=0.0002) and scored 4 dpi.](image)

The glucocorticoid inducible system utilizes the regulatory mechanism of steroids to regulate expression. It consists of two components, a GVG gene which contains a GAL4 DNA binding domain, a VP16 transactivation domain and a glucocorticoid receptor (GR). The second component of this system is the gene of interest (**REF6**) fused to a promoter containing six tandem copies of GAL4 upstream activation sequence (6XGAL4 UAS). When treated with a hormone, in our case dexamethasone (DEX), it results in the nuclear localization and binding of GAL4, the activation of transcription by VP16, and the overexpression of our gene of interest under the control of strong 35S promoter (Aoyama and Chua, 1997). The glucocorticoid
inducible system allowed us to limit the induction of REF6 expression until the plants were healthy looking adults. Unfortunately, the glucocorticoid inducible system is leaky and leads to early flowering in our overexpression lines. Western blot analysis of our REF6OX lines show that we were able to successfully clone and express REF6 (Figure 2.7). We have currently generated homozygote lines expressing WT REF6 and all the mutants in the ref6 mutant background we hope to test their role in defense against pathogen infection in the near future.

Figure 2.5: Inducible overexpression of REF6 using the glucocorticoid inducible system

![Figure 2.5](image_url)

**Full Length WT REF6**

<table>
<thead>
<tr>
<th>REF6</th>
<th>JmjN</th>
<th>JmjC</th>
<th>Coiled Coil</th>
<th>Phospho site</th>
<th>Zinc Fingers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>19-60</td>
<td>200-369</td>
<td>514-535</td>
<td>S</td>
<td></td>
</tr>
</tbody>
</table>

**Six REF6 Phospho Mutants**

- S683A
- S683E
- S683D
- S1092A
- S1092E
- S1092D

**REF6-CΔ4ZnF – Zinc Finger Domain Deleted**

<table>
<thead>
<tr>
<th>REF6</th>
<th>JmjN</th>
<th>JmjC</th>
<th>Coiled Coil</th>
<th>Phospho site</th>
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Figure 2.6: REF6 overexpression lines

![Figure 2.6](image_url)
Mass spectrometry analysis of overexpression line

We treated REF6 overexpression line with 50uM of dexamethasone for 4hrs. The leaves were cut and flash frozen and the protein extracted from the plant tissue. The extracted proteins were analyzed by mass spectrometry and compared with non induced plants. The mass spectrometry results showed that 449 proteins changed in abundance after DEX induction and of these 430 increased in abundance while 16 reduced in abundance. This is very interesting because it shows that REF6 may play an activatory role during defense signaling. Of the highly overrepresented proteins we observed MAP kinases, cell wall related proteins and hormone related proteins.
Discussion

REF6 is a transcription factor with jumonji-domains known to play a role in flowering. We observed from our mass spectrometry data that REF6 is rapidly phosphorylated in two sites following 5 mins of BTH treatment suggesting that besides its role in flowering, REF6 might play a role in plant defense against pathogen infection.

To determine the role of REF6 in defense we subjected $ref6$ mutants to infection with $Pto$ DC3000, in the presence and absence of BTH treatment to determine REF6 role in PTI and induced resistance (IR) respectively. We observed that REF6 showed enhanced disease susceptibility phenotype when infected without priming and enhanced disease resistance when primed. This suggests that REF6 is capable of acting as both as an activator and an inhibitor of defense. As an activator, REF6 is required for defense against DC3000 however it is required to inhibit excessive induced resistance which can be energy costly and detrimental to plant health.

To further determine REF6 function, we infected $ref6$ with three isolates of Hyaloperanospora: $Hpa$ Emwa1, Hpa Noco2, and $Hpa$ Emco5. We observed that of the three strains, REF6 is required for defense against $Hpa$ Emwa1 and therefore functions in PTI and RPP4-mediated ETI. To determine if REF6 is involved in RPM1-mediated ETI, we infected ref6 with $Pto$ AvrRpm1 and determined that REF6 is not required for RPM1 mediated ETI.

Given that BTH induced enhanced resistance in $ref6$, we decided to test $ref6$ IR phenotype in plants that are primed with flg22. Interestingly, flg22 did not successfully prime $ref6$ instead $ref6$ showed impaired flg22 response, further complicating REF6 role in defense.

We sought to see if overexpression of $REF6$ will give us an idea of REF6’s mode of action. Due to potential complications in constitutive overexpression of REF6, we overexpressed $REF6$ in a glucocorticoid (DEX) inducible system. Our preliminary results from mass spectrometry analysis of DEX treated plants compared to untreated plants revealed that REF6 induced the increase in abundance of majority of the proteins leaving only 16 out of 439 proteins
that reduced in abundance. This reveals that REF6 is an activator of expression. This makes sense because H3K27me3 are inhibitory methylation marks placed by histone methyl transferases and REF6 functions in the demethylation of H3K27me3 (Chen et al., 2011, Lu et al., 2011). REF6 is therefore a transcription activator that may positively regulate proteins that function in both the activation and inhibition of defense thereby resulting in complex phenotypes.

We have currently created phosphodead, phosphomimcs and deletion mutants of \textit{REF6} with the hope that these lines can aid us in understanding REF6’s complex role in defense and to help us untangle where REF6 fits in defense and hormone signaling.
Materials and methods

Plant growth conditions and treatments

All plants used with the exception of the Ws lines used as Hpa controls, were Arabidopsis thaliana Col-0 genetic background. WT and all knockout mutant seeds were obtained from the Arabidopsis Biological Resource center (ABRC); all mutant lines used are listed in Appendix Table 4). All WT and mutant plants used for mass spectrometric analysis and Pseudomonas syringae pv. tomato DC3000 bacterial infection assay were grown on 3:1 soil (sunshine professional) to vermiculite (Therm-O-Rock) mixture in 11-h light/13-hours dark condition at 23°C for 4-5 weeks in a Percival intellus control system growth chamber.

For BTH induced changes in the total proteome, and phosphoproteome, 5-week-old WT and npr1-1 plants were sprayed with 300uM BTH (Chem Service PS-2212), 0.1% DMSO and 0.03% Silwet L-77 surfactant (GE Silicones, VIS-01) for 5 minutes. Control plants were sprayed with 0.1% DMSO and 0.03% Silwet for 5 minutes. After treatment all above ground tissue were cut, flash frozen with liquid nitrogen, and subjected to protein extraction for mass spectrometric analysis.

Protein Extraction and tryptic digestion

For each sample prepared for mass spectrometric analysis, approximately 2.5g of frozen leaves were ground in liquid nitrogen using a pre-chilled ceramic mortar and pestle for 15 min. The crushed samples were transferred to a 50ml conical tube and resuspended in -20°C pre-chilled methanol containing 0.2mM Na3VO4. The samples were incubated at -20°C for 15 minutes and then centrifuged at 4,000g for 5 min at 4°C. The Supernatant was discarded and the pellet was washed two additional times with the pre-chilled methanol containing 0.2mM Na3VO4, each wash consisted of a -20°C incubation for 15 minutes and a 5 mins centrifugation step. The pellets were then washed three times with -20°C chilled acetone using the same incubation and
centrifugation process. The sample pellets were dried in an Eppendorf Vacufuge Concentrator at 4°C. Proteins were extracted from the dry pellets by the addition 1ml of 0.2% RapiGest, 1mM EDTA, and 50mM HEPES. The mixture was sonicated at 4°C for 1 minute using the sonicator then incubated on ice for 15 minutes. The digested samples were reduced by treatment with Tris(2-carboxyethyl)phosphine TCEP (Fisher, AC36383) for 5 minutes at 94°C. 1mg of each sample was treated with 1ug/ul of Trypsin and shaken overnight at 37°C using. The trypsinized samples were alkylated with 2.5mM iodoacetamide (FisherAC12227) for 15 minutes at 37°C in the dark. The samples were then centrifuged at 16,100g for 15 min. the Supernatant was filtered with 0.22uM spin column (Fisher# 07-200-386) by centrifugation for 15 min. The flow-through was iTRAQ labeled for LC-MS/MS analysis.

The iTRAQ labeling and phosphopeptide enrichment

Samples used for total proteome and phosphoproteome analysis were subjected to iTRAQ labeling. 1mg of each digested sample was mixed with 1M Hepes buffer (pH 7.2) and vacufuged to concentrate volume to approximately 50ul. 1ml of 100% IPA was added to each sample. 185ul of IPA was added to each tube of iTRAQ (4-plex iTRAQ, Applied Biosystems 4352135) and 100ul of the mixture was added to each sample. The samples were sonicated in a bath type sonicator for 15 minutes at 4°C. 500ul of water was added, and another 100ul of iTRAQ/IPA mixture was added to each sample. The samples were sonicated again and incubated at room temperature for 1 hour. The samples were dried by Eppendorf Vacufuge Concentrator then resuspended in 500ul of H2O and merged. The merged samples were treated with 2% TFA to precipitate RapiGest, incubated at room temperature for 1 hour, then centrifuged at 16,100g for 15 minutes at room temperature. The supernatant was collected and centrifuged through a 0.22 uM filter. And used for total proteome mass spec analysis. Samples that were used for phosphoproteome analysis were further subjected to phosphopeptide enrichment by adding 100ul
of 1% colloidal CeO$_2$ and vortexing for 30 seconds. The samples were centrifuged at 500g for 1 min and the supernatant was discarded. The CeO$_2$ pellet was washed with 1ml of 1% TFA/mg protein and vortexed for 5 secs. The samples were centrifuged again for 1 min and the supernatant discarded. 100 ul of 100mM (NH$_4$)$_2$HP0$_4$/1M NH$_3$.H$_2$O, pH 9.5 was added to the mix and vortexed for 30 sec. 3% TFA (pH 1.8) was added and vortexed for 5 seconds the centrifuges at 16,100g for 1 minute. The supernatant was used for mass spectrometry analysis

**LC-MS/MS analysis**

An Agilent 1100 HPLC system (Agilent Technologies, Wilmington, DE) delivered a flow rate of 300 nL min$^{-1}$ to a 3-phase capillary chromatography column through a splitter. Using a custom pressure cell, 5 µm Zorbax SB-C18 (Agilent) was packed into fused silica capillary tubing (200 µm ID, 360 µm OD, 20 cm long) to form the first dimension reverse phase column (RP1). A 5 cm long strong cation exchange (SCX) column packed with 5 µm PolySulfoethyl (PolyLC) was connected to RP1 using a zero dead volume 1 µm filter (Upchurch, M548) attached to the exit of the RP1 column. A fused silica capillary (100 µm ID, 360 µm OD, 20 cm long) packed with 5 µm Zorbax SB-C18 (Agilent) was connected to SCX as the analytical column (RP2). The electro-spray tip of the fused silica tubing was pulled to a sharp tip with the inner diameter smaller than 1 µm using a laser puller (Sutter P-2000). The peptide mixtures were loaded onto the RP1 column using the custom pressure cell. Columns were not re-used. Peptides were first eluted from the RP1 column to the SCX column using a 0 to 80% acetonitrile gradient for 150 minutes. The peptides were fractionated by the SCX column using a series of salt gradients (from 10 mM to 1 M ammonium acetate for 20 minutes), followed by high resolution reverse phase separation using an acetonitrile gradient of 0 to 80% for 120 minutes.
Spectra were acquired on LTQ linear ion trap tandem mass spectrometers (Thermo Electron Corporation, San Jose, CA) employing automated, data-dependent acquisition. The mass spectrometer was operated in positive ion mode with a source temperature of 150 °C.

The full MS scan range of 400-2000 m/z was divided into 3 smaller scan ranges (400-800, 800-1050, 1050-2000) to improve the dynamic range. Both CID (Collision Induced Dissociation) and PQD (Pulsed-Q Dissociation) scans of the same parent ion were collected for protein identification and quantitation. Each MS scan was followed by 4 pairs of CID-PQD MS/MS scans of the most intense ions from the parent MS scan. A dynamic exclusion of 1 minute was used to improve the duty cycle of MS/MS scans. About 20,000 MS/MS spectra were collected for each salt step fractionation.

The raw data was extracted and searched using Spectrum Mill (Agilnet). The CID and PQD scans from the same parent ion were merged together. MS/MS spectra with a sequence tag length of 1 or less were considered as poor spectra and discarded. The rest of the MS/MS spectra were searched against the International Protein Index (IPI) Arabidopsis database. The enzyme parameter was limited to full tryptic peptides with a maximum miscleavage of 1. All other search parameters were set to SpectrumMill’s default settings (carbamidomethylation of cysteines, iTRAQ modification, +/- 2.5 Da for precursor ions, +/- 0.7 Da for fragment ions, and a minimum matched peak intensity of 50%). A concatenated forward-reverse database was constructed to calculate the in-situ false discovery rate (FDR). We dynamically assign cutoff scores for each dataset to maintain our protein FDR less than 1%. Proteins shared common peptides are grouped into protein groups to address the database redundancy issue. The proteins within the same group share the same set or subset of unique peptides.
Relative protein quantitation was performed by calculating the iTRAQ reported ion intensity ratios. Protein iTRAQ intensities were calculated by summing the peptide iTRAQ intensities from each protein group. Peptides shared among different

**Bacterial infection assays**

4-5 weeks-old *Arabidopsis thaliana* (COL-0) plants and T-DNA insertion mutant lines were sprayed with 0.1% DMSO, 0.03% silwet (control) or 0.1% DMSO, 0.03% and 300uM BTH (IR). After 3 days of priming the plants were injected with *Pseudomonas syringae pv. tomato* (*Pto*) DC3000 at a concentration of OD$_{600}$ = 0.0002, using a needleless syringe. Bacteria growth was scored 0, 2 and 4 days post infection.

For the *Hyaloperanospora arabidopsisidis infection*, 2 wks old seedlings were spray infected with sporangiophores, and the spores per ml of infected material was scored 4 or 5 dpi.

For the *Botrytis Cinerea* infection, leaves form 4-5 week-old plants were excised from the plants and placed on agarose media. 5ul of *Botrytis Cinerea* grape isolate was dropped on the leaves and the lesions were measured 4 days after infection.

**STRING analysis**

All the proteins that changed after BTH treatment in the total proteome, phosphoproteome and npr1-1 proteome were combined and entered into the SPRING online database.
**APPENDIX**

Appendix Table 1: List of proteins that changed in abundance following 5mins BTH treatment. AGI code, average change, p-value and annotation from TAIR are shown on the table. The results are based on 3 biological replicates. Changes greater than 1.5 fold with a p-value of ≤ 0.1 were considered to be significant.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average WT</th>
<th>BTH/Control 5 mins</th>
<th>P-value</th>
<th>Annotation</th>
</tr>
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<tbody>
<tr>
<td>AT5G17550</td>
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<td>CYP86A2</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Cytochrome P450 86A2</td>
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<td>Pentatricopeptide (PPR) repeat-containing protein</td>
</tr>
<tr>
<td>AT5G47720</td>
<td>1.9E+00</td>
<td>0.06</td>
<td></td>
<td>Isoform 2 of Probable acetyl-CoA acetyltransferase, cytosolic 2</td>
</tr>
</tbody>
</table>
Appendix Table 1: List of proteins that changed in abundance following 5mins BTH treatment, Continued.

<table>
<thead>
<tr>
<th>AGI</th>
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<th>Annotation</th>
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### Appendix Table 1: List of proteins that changed in abundance following 5mins BTH treatment, Continued.

<table>
<thead>
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<th>AGI</th>
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<th>Annotation</th>
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<td>Histone H2B.6</td>
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</table>
Appendix Table 1: List of proteins that changed in abundance following 5mins BTH treatment, Continued.

<table>
<thead>
<tr>
<th>AGI</th>
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<th>P-value</th>
<th>Annotation</th>
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<tbody>
<tr>
<td>AT1G76690</td>
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<td>AT3G25760</td>
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<tr>
<td>AT1G14000</td>
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<td>VIK (VH1-INTERACTING KINASE)</td>
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<td>Ankyrin repeat family protein</td>
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<td>AT5G61520</td>
<td>6.3E-01</td>
<td>0.02</td>
<td>Isoform 1 of Sugar transport protein 3</td>
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<tr>
<td>AGI</td>
<td>Average WT</td>
<td>BTH/Control 5 mins</td>
<td>P-value</td>
</tr>
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<td>AT1G01610</td>
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<td>AT4G30020</td>
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<td>AT4G08870</td>
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### Appendix Table 1: List of proteins that changed in abundance following 5mins BTH treatment, Continued.

<table>
<thead>
<tr>
<th>AGI</th>
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<th>P-value</th>
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<tbody>
<tr>
<td>AT5G61500</td>
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<td>Autophagy-related protein 3</td>
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<td>AT2G07180</td>
<td>5.9E-01</td>
<td>0.03</td>
<td>Protein kinase</td>
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<td>AT3G49120</td>
<td>5.9E-01</td>
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<td>PRXCB Peroxidase 34 precursor</td>
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<tr>
<td>AT2G27040</td>
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<td>AGO4 AGO4 (ARGONAUTE 4)</td>
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<tr>
<td>AT5G26340</td>
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<td>AT1G24050</td>
<td>5.6E-01</td>
<td>0.08</td>
<td>Similar to unknown protein</td>
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</table>
Appendix Table 1: List of proteins that changed in abundance following 5mins BTH treatment, Continued.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average WT BTH/Control 5 mins</th>
<th>P-value</th>
<th>Annotation</th>
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<tbody>
<tr>
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<tr>
<td>AT1G14250</td>
<td>5.4E-01</td>
<td>0.04</td>
<td>Nucleoside phosphatase family protein</td>
</tr>
<tr>
<td>AT2G17230</td>
<td>5.2E-01</td>
<td>0.09</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>AT1G15500</td>
<td>5.1E-01</td>
<td>0.00</td>
<td>Chloroplast ADP,ATP carrier protein 2, chloroplast precursor</td>
</tr>
<tr>
<td>AT4G17470</td>
<td>5.0E-01</td>
<td>0.03</td>
<td>Palmitoyl protein thioesterase family protein</td>
</tr>
<tr>
<td>AT1G77710</td>
<td>4.9E-01</td>
<td>0.04</td>
<td>Probable ubiquitin-fold modifier 1 precursor</td>
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</table>
### Appendix Table 1: List of proteins that changed in abundance following 5mins BTH treatment, Continued.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average WT BTH/Control 5 mins</th>
<th>P-value</th>
<th>Annotation</th>
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<tbody>
<tr>
<td>AT5G59730</td>
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<tr>
<td>AT2G23930</td>
<td>4.7E-01</td>
<td>0.05</td>
<td>SNRNP-G Probable small nuclear ribonucleoprotein G</td>
</tr>
<tr>
<td>AT2G19830</td>
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<td>SNF7 family protein</td>
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<tr>
<td>AT1G16030</td>
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<tr>
<td>AT2G37770</td>
<td>3.0E-01</td>
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<td>Oxidoreductase</td>
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Appendix Table 2: List of phosphoproteins and phosphopeptides observed after 5 mins BTH treatment. AGI code, average change in phosphorylation, p-value, annotation from TAIR, and phosphopeptide sequence are shown on the table. The results are based on 3 biological replicates. Changes greater than 1.5 fold, with a p-value of ≤ 0.1, and no significant changes observed in total protein abundance levels was considered significant.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average change in Phosphorylation of WT BTH/Control 5 mins</th>
<th>p-value</th>
<th>Annotation</th>
<th>Peptide Sequence</th>
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<tbody>
<tr>
<td>AT2G24940</td>
<td>3.2E+00</td>
<td>0.00</td>
<td>ATMAPR2 Putative steroid- binding protein 3</td>
<td>NEEDVsPSLEGLTEK</td>
</tr>
<tr>
<td>AT4G39400</td>
<td>2.5E+00</td>
<td>0.07</td>
<td>BRI1 Protein BRASSINOSTEROID INSENSITIVE 1 precursor</td>
<td>EAELEmYAEGHGNsGDR</td>
</tr>
<tr>
<td>AT1G31440</td>
<td>2.3E+00</td>
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### Appendix Table 2: List of phosphoproteins and phosphopeptides observed after 5 mins BTH treatment, Continued

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average change in Phosphorylation of WT BTH/Control 5 mins</th>
<th>p-value</th>
<th>Annotation</th>
<th>Peptide Sequence</th>
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<tr>
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### Appendix Table 2: List of phosphoproteins and phosphopeptides observed after 5 mins BTH treatment, Continued

<table>
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<th>AGI</th>
<th>Average change in Phosphorylation of WT BTH/Control 5 mins</th>
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<td>AT5G55300</td>
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<td>IIKDEsDDETPISS mFR</td>
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Appendix Table 2: List of phosphoproteins and phosphopeptides observed after 5 mins BTH treatment, Continued

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average change in Phosphorylation of WT BTH/Control 5 mins</th>
<th>p-value</th>
<th>Annotation</th>
<th>Peptide Sequence</th>
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<tbody>
<tr>
<td>AT5G47690</td>
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Appendix Table 2: List of phosphoproteins and phosphopeptides observed after 5 mins BTH treatment, Continued

<table>
<thead>
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<tr>
<td>AT2G42760</td>
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<td>Similar to unknown protein</td>
<td>smSDLEYEELK</td>
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<td>AT3G55460</td>
<td>6.3E-01</td>
<td>0.03</td>
<td>SCL30 SCL30 (SC35-like splicing factor 30)</td>
<td>RSysPGYEGAAA</td>
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<td>AAPDR</td>
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<td>PHOT1 Phototropin-1</td>
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<tr>
<td></td>
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</table>
### Appendix Table 2: List of phosphoproteins and phosphopeptides observed after 5 mins BTH treatment, Continued

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average change in Phosphorylation of WT BTH/Control 5 mins</th>
<th>p-value</th>
<th>Annotation</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT5G54270</td>
<td>6.2E-01</td>
<td>0.08</td>
<td>LHC B3 LHC B3 (LIGHT-HARVESTING CHLOROPHYLL BINDING PROTEIN 3)</td>
<td>DVVSGLsPK</td>
</tr>
<tr>
<td>AT3G05420</td>
<td>6.1E-01</td>
<td>0.03</td>
<td>ACBP 4 ACBP 4 (ACYL-COA BINDING PROTEIN 4, ACYL-COA-BINDING DOMAIN 4)</td>
<td>DIESEVEVsQEGR</td>
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<tr>
<td>AT5G20280</td>
<td>6.1E-01</td>
<td>0.03</td>
<td>ATSPS1F ATSPS1F (sucrose phosphate synthase 1F)</td>
<td>INsAESME LWASQQK</td>
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<tr>
<td>AT2G04880</td>
<td>6.1E-01</td>
<td>0.00</td>
<td>ZAP1 Isoform 2 of WRKY transcription factor 1</td>
<td>DYNsPtAK</td>
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</tbody>
</table>
Appendix Table 2: List of phosphoproteins and phosphopeptides observed after 5 mins BTH treatment, Continued

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average change in Phosphorylation of WT BTH/Control 5 mins</th>
<th>p-value</th>
<th>Annotation</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G80930</td>
<td>0.05</td>
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<td>MIF4G domain-containing protein</td>
<td>VRRVsDDEDR</td>
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<tr>
<td>AT1G67580</td>
<td>6.1E-01</td>
<td>0.05</td>
<td>Protein kinase family protein</td>
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<tr>
<td>AT2G45540</td>
<td>6.0E-01</td>
<td>0.05</td>
<td>WD-40 repeat family protein</td>
<td>QTCATNAVAAsPS PLK</td>
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<tr>
<td>AT1G01960</td>
<td>5.8 E-01</td>
<td>0.03</td>
<td>Embryo sac development arrest 10</td>
<td>SDSQsELSSGNS DALAIEQR</td>
</tr>
<tr>
<td>AT5G24430</td>
<td>5.8E-01</td>
<td>0.09</td>
<td>Calcium-dependent protein kinase</td>
<td>DEPIPEDSEDVVV DHGGDsGGGER</td>
</tr>
<tr>
<td>AT2G26910</td>
<td>5.8E-01</td>
<td>0.07</td>
<td>PDR4 Probable pleiotropic drug resistance protein 4</td>
<td>StSFKDEIEDEEE LR</td>
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</table>
Appendix Table 2: List of phosphoproteins and phosphopeptides observed after 5 mins BTH treatment, Continued

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average change in Phosphorylation of WT BTH/Control 5 mins</th>
<th>p-value</th>
<th>Annotation</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT4G26530</td>
<td>5.7E-01</td>
<td>0.01</td>
<td>Fructose-bisphosphate aldolase</td>
<td>GILAADESTGtIG K</td>
</tr>
<tr>
<td>AT3G05900</td>
<td>5.5E-01</td>
<td>0.04</td>
<td>Neurofilament protein-related</td>
<td>ETEGDVPsPADV IEK</td>
</tr>
<tr>
<td>AT3G43300</td>
<td>5.4E-01</td>
<td>0.01</td>
<td>ATMIN7 ATMIN7 (ARABIDOPSIS THALIANA HOPM INTERACTOR 7)</td>
<td>SSVAEVTVPsSP YK</td>
</tr>
<tr>
<td>AT1G21630</td>
<td>5.1E-01</td>
<td>0.06</td>
<td>Calcium-binding EF hand family protein</td>
<td>EGEDVSFsDADS K</td>
</tr>
<tr>
<td>AT1G59610</td>
<td>3.8E-01</td>
<td>0.06</td>
<td>ADL3 Dynamin-2B</td>
<td>AAAASSWSDNS GTEsSPR</td>
</tr>
<tr>
<td>AT1G20670</td>
<td>3.4E-01</td>
<td>0.07</td>
<td>DNA-binding bromodomain-containing protein</td>
<td>QDsDDEEPQSQQ QQQQQPK</td>
</tr>
</tbody>
</table>
## Appendix Table 2: List of phosphoproteins and phosphopeptides observed after 5 mins BTH treatment, Continued

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average change in Phosphorylation of WT BTH/Control 5 mins</th>
<th>p-value</th>
<th>Annotation</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G20670</td>
<td>1.9E-01</td>
<td>0.09</td>
<td>DNA-binding bromodomain-containing protein</td>
<td>TASEIsADALIPG DSSNK</td>
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<tr>
<td>IPI00540432</td>
<td>1.5E-01</td>
<td>0.10</td>
<td>Topoisomerase-like protein</td>
<td>NDPNYDGEDAYDGLVDPVSDPLNDYK</td>
</tr>
</tbody>
</table>
Appendix Table 3: List of proteins that changed in abundance in npr1-1 mutant compared to WT following 5mins BTH treatment. AGI code, average change, p-value and annotation from TAIR are shown on the table. The results are based on 3 biological replicates. Changes greater than 1.5 fold with a p-value of ≤ 0.1 were considered to be significant.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average npr1-1 BTH/WT BTH</th>
<th>p-value</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT4G38630</td>
<td>5.9E+00</td>
<td>0.04</td>
<td>AT-MCB1 26S proteasome non-ATPase regulatory subunit 4</td>
</tr>
<tr>
<td>AT5G46070</td>
<td>3.8E+00</td>
<td>0.01</td>
<td>GTP binding</td>
</tr>
<tr>
<td>AT2G05100</td>
<td>3.4E+00</td>
<td>0.07</td>
<td>LHC2.1 LHC2.1 (Photosystem II light harvesting complex protein 2.1)</td>
</tr>
<tr>
<td>G33670</td>
<td>3.0E+00</td>
<td>0.04</td>
<td>L-galactose dehydrogenase (L-GalDH)</td>
</tr>
<tr>
<td>AT3G56010</td>
<td>2.7E+00</td>
<td>0.10</td>
<td>Similar to conserved hypothetical protein</td>
</tr>
<tr>
<td>AT1G52220</td>
<td>2.2E+00</td>
<td>0.07</td>
<td>Similar to TMP14 (THYLAKOID MEMBRANE PHOSPHOPROTEIN OF 14 KDA)</td>
</tr>
</tbody>
</table>
Appendix Table 3: List of proteins that changed in abundance in *npr1-1* mutant compared to WT following 5mins BTH treatment.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average ( \frac{npr1-1}{BTH/WT} )</th>
<th>p-value</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT3G54760</td>
<td>2.2E+00</td>
<td>0.06</td>
<td>Dentin sialophosphoprotein-related</td>
</tr>
<tr>
<td>AT4G36250</td>
<td>2.1E+00</td>
<td>0.01</td>
<td>ALDH3F1 Aldehyde dehydrogenase 3F1</td>
</tr>
<tr>
<td>AT3G54900</td>
<td>2.1E+00</td>
<td>0.00</td>
<td>CXIP1 Monothiol glutaredoxin-S14, chloroplast precursor</td>
</tr>
<tr>
<td>AT5G48900</td>
<td>1.9E+00</td>
<td>0.09</td>
<td>Probable pectate lyase 20 precursor</td>
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<tr>
<td>AT1G11650</td>
<td>1.7E+00</td>
<td>0.02</td>
<td>ATRBP45B ATRBP45B</td>
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<tr>
<td>AT4G13340</td>
<td>1.6E+00</td>
<td>0.01</td>
<td>Leucine-rich repeat family protein</td>
</tr>
</tbody>
</table>
Appendix Table 3: List of proteins that changed in abundance in *npr1-1* mutant compared to WT following 5mins BTH treatment.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average ( \frac{npr1-1}{BTH} )</th>
<th>p-value</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G24490</td>
<td>1.5E+00</td>
<td>0.08</td>
<td>ALB4 Protein ARTEMIS, chloroplast precursor</td>
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<tr>
<td>AT4G34050</td>
<td>1.5E+00</td>
<td>0.04</td>
<td>Probable caffeoyl-CoA O-methyltransferase</td>
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<tr>
<td>AT2G24420</td>
<td>1.5E+00</td>
<td>0.02</td>
<td>DNA repair ATPase-related</td>
</tr>
<tr>
<td>AT3G22960</td>
<td>6.6E-01</td>
<td>0.02</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>AT4G20330</td>
<td>6.6E-01</td>
<td>0.02</td>
<td>Transcription initiation factor-related</td>
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<tr>
<td>AT1G13280</td>
<td>6.5E-01</td>
<td>0.01</td>
<td>AOC4 Allene oxide cyclase 4, chloroplast precursor</td>
</tr>
</tbody>
</table>
Appendix Table 3: List of proteins that changed in abundance in *npr1-1* mutant compared to WT following 5mins BTH treatment.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average ( npr1-1 ) BTH/WT</th>
<th>p-value</th>
<th>Annotation</th>
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<tr>
<td>AT5G12040</td>
<td>6.4E-01</td>
<td>0.05</td>
<td>Carbon-nitrogen hydrolase family protein</td>
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<tr>
<td>AT3G13580</td>
<td>6.4E-01</td>
<td>0.08</td>
<td>60S ribosomal protein L7-4</td>
</tr>
<tr>
<td>AT5G61640</td>
<td>6.4E-01</td>
<td>0.05</td>
<td>PMSR1 PMSR1 (PEPTIDEMETHIONINE SULFOXIDE REDUCTASE 1)</td>
</tr>
<tr>
<td>AT5G61510</td>
<td>6.4E-01</td>
<td>0.03</td>
<td>NADP-dependent oxidoreductase</td>
</tr>
<tr>
<td>AT2G01140</td>
<td>6.4E-01</td>
<td>0.04</td>
<td>Probable fructose-bisphosphate aldolase 3, chloroplast precursor</td>
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<tr>
<td>AT5G27540</td>
<td>6.3E-01</td>
<td>0.01</td>
<td>EMB2473 EMB2473 (EMBRYO DEFECTIVE 2473)</td>
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</table>
Appendix Table 3: List of proteins that changed in abundance in *npr1-1* mutant compared to WT following 5mins BTH treatment.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average <em>npr1-1</em> BTH/WT BTH</th>
<th>p-value</th>
<th>Annotation</th>
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<tbody>
<tr>
<td>AT5G03650</td>
<td>6.2E-01</td>
<td>0.10</td>
<td>SBE2.2 1, 4-alpha-glucan branching enzyme protein soform SBE2.2</td>
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<tr>
<td>AT1G52410</td>
<td>6.2E-01</td>
<td>0.07</td>
<td>TSA1 TSA1 (TSK-ASSOCIATING PROTEIN 1)</td>
</tr>
<tr>
<td>AT1G45201</td>
<td>5.9E-01</td>
<td>0.04</td>
<td>Similar to lipase class 3 family protein</td>
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<tr>
<td>AT5G27120</td>
<td>5.8E-01</td>
<td>0.02</td>
<td>Probable nucleolar protein NOP5-1</td>
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<tr>
<td>AT5G64430</td>
<td>5.7E-01</td>
<td>0.06</td>
<td>Octicosapeptide/Phox/Bem1p (PB1) domain-containing protein</td>
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<tr>
<td>AT5G56360</td>
<td>5.7E-01</td>
<td>0.08</td>
<td>Calmodulin-binding protein</td>
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</table>
Appendix Table 3: List of proteins that changed in abundance in *npr1-1* mutant compared to WT following 5mins BTH treatment.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average <em>npr1-1</em> BTH/WT BTH</th>
<th>p-value</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT3G23300</td>
<td>5.5E-01</td>
<td>0.06</td>
<td>Dehydration-responsive protein-related</td>
</tr>
<tr>
<td>AT1G52400</td>
<td>5.4E-01</td>
<td>0.03</td>
<td>BGL1 Beta-glucosidase homolog precursor</td>
</tr>
<tr>
<td>AT5G43960</td>
<td>5.4E-01</td>
<td>0.06</td>
<td>Nuclear transport factor 2 (NTF2) family protein</td>
</tr>
<tr>
<td>AT3G22230</td>
<td>5.0E-01</td>
<td>0.06</td>
<td>60S ribosomal protein L27-2</td>
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<tr>
<td>AT3G59920</td>
<td>4.6E-01</td>
<td>0.02</td>
<td>ATGDI2 ATGDI2 (RAB GDP DISSOCIATION INHIBITOR 2)</td>
</tr>
<tr>
<td>AT5G24770</td>
<td>4.6E-01</td>
<td>0.03</td>
<td>VSP2 Vegetative storage protein 2 precursor</td>
</tr>
</tbody>
</table>
Appendix Table 3: List of proteins that changed in abundance in npr1-1 mutant compared to WT following 5mins BTH treatment.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Average BTH/WT BTH npr1-1</th>
<th>P-value</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT3G25770</td>
<td>4.1E-01</td>
<td>0.05</td>
<td>AOC2 Allene oxide cyclase 2, chloroplast precursor</td>
</tr>
<tr>
<td>AT2G05380</td>
<td>4.0E-01</td>
<td>0.05</td>
<td>GRP3S GRP3S (GLYCINE-RICH PROTEIN 3 SHORT ISOFORM)</td>
</tr>
<tr>
<td>AT5G16440</td>
<td>3.6E-01</td>
<td>0.01</td>
<td>IPP1 IPP1 (ISOPENTENYL DIPHOSPHATE ISOMERASE 1)</td>
</tr>
<tr>
<td>AT5G10470</td>
<td>2.6E-01</td>
<td>0.04</td>
<td>Kinesin motor protein-related</td>
</tr>
<tr>
<td>AT3G57030</td>
<td>2.3E-01</td>
<td>0.09</td>
<td>Strictosidine synthase family protein</td>
</tr>
<tr>
<td>AT2G37190</td>
<td>1.8E-02</td>
<td>0.09</td>
<td>60S ribosomal protein L12-1</td>
</tr>
<tr>
<td>IPI00533874</td>
<td>1.2E-03</td>
<td>0.05</td>
<td>Hypothetical protein</td>
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Appendix Table 4: List of proteins selected for further studies. AGI code, stock name (mutant line), annotation from TAIR and changes observed via mass spectrometry are shown on the table. All selected proteins are based on results from 3 biological replicates and have p. value of ≤ 0.1.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Mutant line</th>
<th>Annotation</th>
<th>Mass Spectrometry observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G01610</td>
<td>Salk_150886C</td>
<td>Glycerol- 3-phosphate acyl transferase 4</td>
<td>Decrease in abundance</td>
</tr>
<tr>
<td>AT1G01960</td>
<td>Salk_044617C</td>
<td>Embryo sac development arrest 10</td>
<td>Dephosphorylated</td>
</tr>
<tr>
<td>AT1G14250</td>
<td>Salk_097863C</td>
<td>GDA1/CD39 Nucleoside phosphate family protein</td>
<td>Decrease in abundance</td>
</tr>
<tr>
<td>AT2G04880</td>
<td>Salk_070989C</td>
<td>Zinc-dependent activator protein-1</td>
<td>Dephosphorylated</td>
</tr>
<tr>
<td>AT3G56370</td>
<td>Salk_038787C</td>
<td>Leucine-rich repeat protein kinase family prorein</td>
<td>Increase in abundance</td>
</tr>
<tr>
<td>AT4G26530</td>
<td>Salk_080758C</td>
<td>Fructose-bisphosphate aldolase, putative</td>
<td>Dephosphorylated</td>
</tr>
<tr>
<td>AT5G16990</td>
<td>Salk_052140C</td>
<td>NADP-dependent oxidoreductase</td>
<td>Decrease in abundance</td>
</tr>
<tr>
<td>AT5G54270</td>
<td>Salk_020314C</td>
<td>LHCBS light-harvesting chlorophyll B-binding protein 3</td>
<td>Dephosphorylated</td>
</tr>
<tr>
<td>AT1G64280</td>
<td>CS3726</td>
<td>Nonexpressor of PR genes 1</td>
<td>Not observed (Control)</td>
</tr>
</tbody>
</table>
Appendix Figure 1: Go annotation of the biological processes the phosphoproteome. Black bars indicate GO annotation of phosphoproteins that change in phosphorylation after BTH treatment. Grey bars indicate GO annotation of the total phosphoproteins observed.

* P<0.05 ** P<0.01 *** P<0.001 **** P<0.0001
Appendix Figure 2: Go annotation of the biological processes of npr1-1 proteome. Black bars indicate GO annotation of proteins that change in abundance after BTH treatment in npr1-1 compared to WT. Grey bars indicate GO annotation of the total proteins observed. * P<0.05 ** P<0.01 *** P<0.001 **** P<0.0001
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