UNIVERSITY OF CALIFORNIA, SAN DIEGO

Redox Regulation by Thioredoxins in Plant Immunity

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by

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The thesis of Yujung Michelle Lee is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2013

I dedicate this thesis to my parents, for their most generous support and love.

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List of Abbreviations

1. ABA: Abscisic acid
2. BTH: Benzo(1,2,3)thiadiazole-7-carbothioic acid
3. DEX: Dexamethasone
4. ET: Ethylene
5. ETI: effector-triggered immunity
6. ICAT: isotope coded affinity tag
7. IR: Induced resistance
8. JA: jasmonic acid
9. LAR: Local acquired resistance
10. PAMP: pathogen associated molecular pattern
11. PTI: PAMP-triggered immunity
12. RT-PCR: reverse transcription polymerase chain reaction
13. SA: salicylic acid
14. SAR: Systemic acquired resistance
15. TRX: Thioredoxin
16. WT: wild type

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ABSTRACT OF THE THESIS

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Professor Steven P. Briggs, Chair

Members of *Arabidopsis thaliana* cytosolic thioredoxins play various roles in plant defense against pathogen infections. Thioredoxin h5 (AtTRXh5) catalyzes the reduction and activation of NPR1, a master regulator of plant systemic acquired resistance (SAR) to various pathogens. Thioredoxin h3 (AtTRXh3) and AtTRXh5 confer plant's sensitivity to victorin, which is an effector secreted by *Cochliobolus victoriae* to establish pathogenesis. To extend the analysis of the roles of AtTRXh3 and AtTRXh5 in defense against both biotrophic and necrotrophic pathogens, we challenged mutant *trxs* to

Pseudomonas syringae and *Botrytis cinerea* infections. We show that although AtTRXh5 is known to catalyze the NPR1 oligomer-to-monomer switch, it is not required for induced resistance (IR) against a biotrophic pathogen. Furthermore, our bioassay results suggest that reduction of NPR1 by AtTRXh5 may be replaced by other thioredoxins in the cytosol, thus enabling plants to mount IR even in the absence of both AtTRXh3 and AtTRXh5. We also found that AtTRXh3 and AtTRXh5 are required for full immunity against *Botrytis cinerea*, a necrotrophic pathogen. Quantitative proteomic approach using isotope coded affinity tag (ICAT)-labeling was utilized to grasp how *Arabidopsis* redoxome alters upon defense elicitation by benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH), a potent analog of salicylic acid. Our analysis suggests that TRXs may inhibit defense-induced redox alterations of proteins, many of which have implications in both salicylic acid and jasmonic acid-mediated defense pathways.

Introduction

Safeguard of natural resources depends on plant immunity

Plants provide us with numerous resources, including medicine, clothing, and most importantly, sustenance. Majority of human nutrition relies on regular consumption of major crop plants, such as wheat, maize, potato, and rice. However, biotic and abiotic stresses such as pathogen infection or drought impose devastating effects on plant health and consequently, diminish net crop yields. Malnutrition remains as one of the most persistent issues in the world- approximately 12.5% of the global population (868 million people) suffer from starvation (FAO, 2013). Shortage and unavailability of crop plants owe to this epidemic and therefore it is clear that the world's food security heavily relies on the general health of major crop plants.

Common pathogens that cause diseases in plants are fungi, nematodes, oomycetes, viruses, and bacteria (Strange & Scott, 2005). Most of these phytopathogens can also be categorized into biotrophs, hemibiotrophs, and necrotrophs depending on their lifestyles upon invasion of their host plants. Biotrophs thrive on living cells, necrotrophs feed on dead tissues, and hemibiotrophs display biotrophic periods followed by a necrotrophic period (Dou & Zhou, 2012). Some examples of severe biotic stresses that plants are under are pathogen infections caused by biotrophic bacteria *Pseudomonas syringae* and necrotrophic fungus *Botrytis cinerea*. Pathogen infections cause plants to develop severe disease symptoms such as bacterial speck, crown gall, wilt, and even death (Abramovitch, Anderson, & Martin, 2006). As a result of pathogen invasions, the crop yields become significantly prone to reduction. The goal of optimizing general plant

health therefore undermines the preservation of our natural resources, and it requires a comprehensive understanding of the complex biological mechanisms in plant immunity.

Two main branches of the plant immune system

The plant immune system is characterized by two-tiered innate immune pathways, with the first tier being microbial- or pathogen-associated molecular pattern (MAMP or PAMP) triggered immunity (PTI) and the second being effector triggered immunity (ETI). During infection, the pattern recognition receptors (PRRs) residing in plant membranes recognize and interact with PAMPs, which are evolutionarily conserved molecules released by the pathogen. PAMPs usually play vital roles in bacterial function, and an example of a PAMP is the flagellin of a bacterium. PAMP recognition is a key event that initiates the PTI, for orchestration of downstream basal defense responses, including mitogen-activated protein kinase kinase (MAPKK) pathways, transcriptional reprogramming, secretion of antimicrobial proteins and callose that strengthens the cell walls (Error! Reference source not found.) (Abramovitch et al., 2006; Asai et al., 2002; oller & Felix, 2009; Dangl & Jones, 2001; Grant, Fisher, Chang, Mole, & Dangl, 2006).

Pathogens, however, are able to bypass the PTI and gain pathogenicity in plants by utilizing a sophisticated secretion system. For instance, gram-negative phytopathogen *Pseudomonas syringae* attaches to the host cell's surface and uses a plasma membrane-spanning protein complex called type III secretion system (T3SS) to unload their effector molecules into the plant cytoplasm (Figure 1). These effector proteins provide assistance to pathogens in gaining virulence inside the host, and they are known to serve numerous

functions, some including modulation of signaling pathways that are vital for facilitating survival and replication of the pathogen (Cornelis, 2000; Ham, Sreelatha, & Orth, 2011).

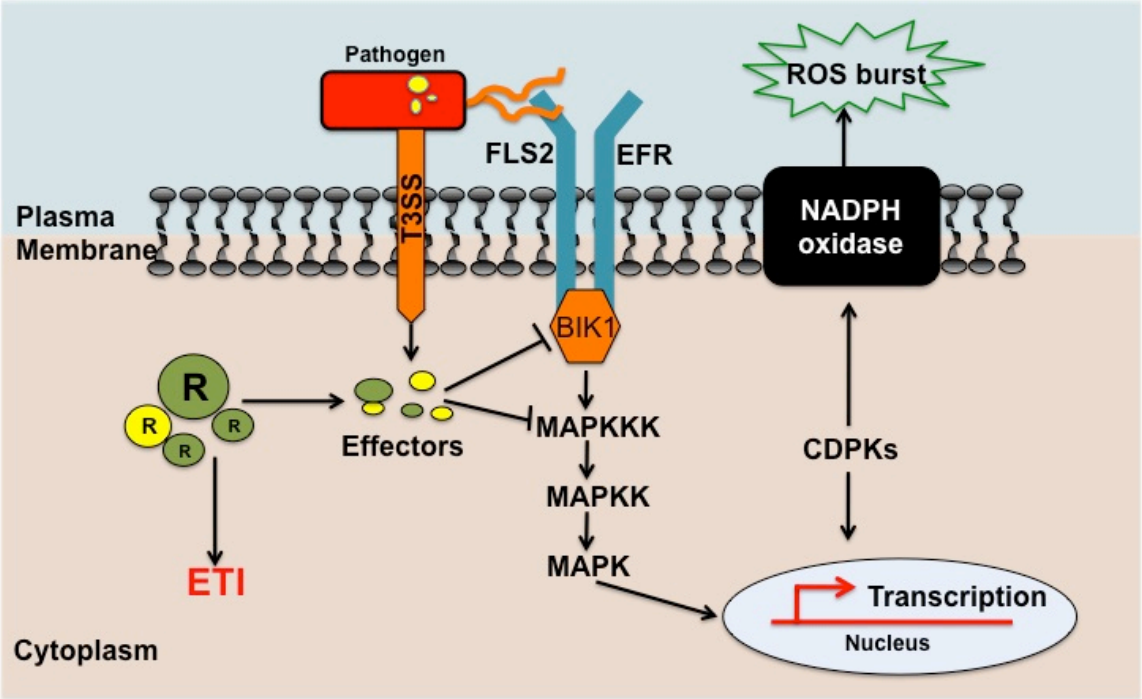


Figure 1. Overview of the plant immune system.

In response to the presence of effector molecules in the plant cytoplasm, plants activate the second branch of immune response, termed effector triggered immunity (ETI). Therefore, ETI is based more on a specific interaction between the plant's disease resistance (R) proteins and pathogen effectors. The characteristic nucleotide binding-leucine rich repeat (NB-LRR) domains of most R-proteins recognize and guard against pathogens that release effectors to disrupt PAMP-signaling pathway. Furthermore, activation of the ETI triggers heightened defense responses, such as hypersensitive cell death (HR) and production of reactive oxygen species (ROS) at the site of infection as means of preventing further infection from taking place (Abramovitch et al., 2006; Boller & Felix, 2009; Jones & Dangl, 2006).

Systemic Acquired Resistance

Another crucial event that takes place upon pathogen challenge is an increase in the level of phytohormone salicylic acid (SA) in local and systemic tissues. This phytohormone serves as a transduction immune signal that activates the expression of various pathogenesis-related (PR) proteins, which are known to have antimicrobial properties that inhibit pathogen spread and growth (Loon & Strien, 1999; Malamy, Carr, Klessig, & Raskin, 1990; Rasmussen, Hammerschmidt, & Zook, 1991). Activation of *PR* genes further triggers plants to develop systemic acquired resistance (SAR), which is a phenomenon during which plants gain systemic resistance throughout their tissues against secondary infection by a diverse group of pathogens. SAR is also a type of induced resistance (IR) in plants, which encompasses both systemic and local acquired resistance to pathogen infection. Furthermore, since SAR is characterized by heightened expression of *PR* genes, these genes often serve as molecular markers of SAR (Durrant & Dong, 2004). Furthermore, exogenous treatments of salicylic acid as well as its synthetic analog, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), have been shown to induce the expression *PR* genes (Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996).

The mechanism through which SA regulates defense response is through the maintenance and degradation of NPR1. In the absence of pathogen attack, false defense activation is impeded by a proteasome-mediated degradation of NPR1 by its paralog, NPR4. During pathogen challenge, however, elevated SA level restricts this NPR1 degradation and facilitates NPR1 accumulation for the activation of SAR (Fu et al., 2012). Furthermore, rise in level of SA induces important redox changes in two of the ten

conserved cysteines of NPR1- absence of pathogen challenge retains NPR1 in the cytoplasm as an oligomeric form held together by its redox-sensitive intermolecular disulphide bonds, while increase in SA upon pathogen infection leads to reduction of NPR1 into its monomeric forms. Monomeric NPR1 then translocates into the nucleus, where it serves as a cofactor to *TGA* transcription factors to initiate the expression of *PR* genes (Mou, Fan, & Dong, 2003; Yasuomi Tada, Steven H. Spoel, Karolina Pajerowska-Mukhtar, Zhonglin Mou, Junqi Song, Chun Wang, Jianru Zuo, 2008) (Figure 2).

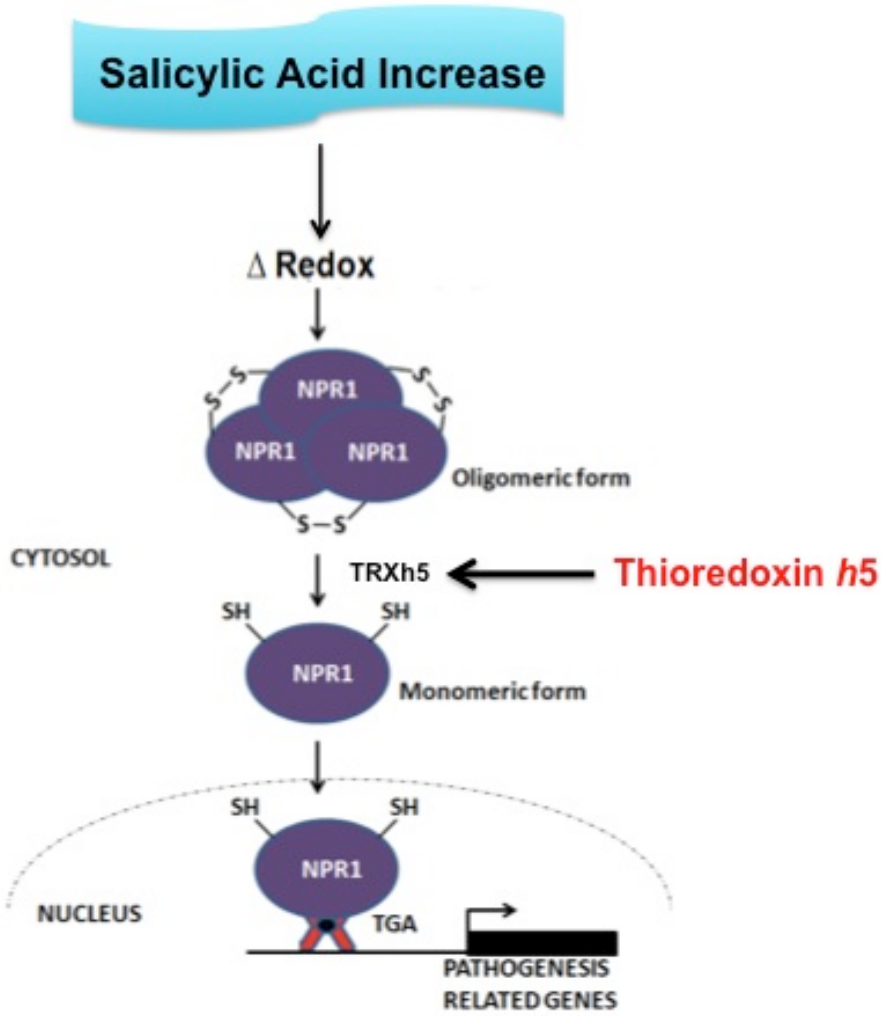


Figure 2. Thioredoxin *h*5 catalyzes the reduction of NPR1 to release its active monomeric form. Increase in cellular SA upon pathogen challenge causes important

Figure 2. Thioredoxin *h*5, Continued. redox changes that facilitate NPR1 oligomer-to-monomer switch. Monomeric NPR1 localizes to the nucleus to activate the transcription of *PR* genes.

Thioredoxins in Plant Immunity

Through pull-down assays, the reducing agent responsible for catalyzing the NPR1 oligomer-to-monomer switch upon SA induction has been identified to be cytosolic (*h*-type) thioredoxin 5 (AtTRXh5) (Figure 2). Another thioredoxin, TRXh3, has also been found to interact with NPR1 but whether it participates in reduction of NPR1 remains unclear. However, both AtTRXh3 and AtTRXh5 are required for full induction of *PR-1* gene, which is required for successful induced resistance (Tada et al., 2008). Therefore, such discovery of a key reducing agent that regulates the activity of an important master regulatory protein of SAR necessitates further extensive studies on which other protein-thioredoxin interactions exist in plant defense against pathogen infection.

Thioredoxins are relatively small (~12 kDa) and ubiquitous oxidoreductases that are known to exist in all living organisms. Structural and enzymatic features generally define thioredoxins: the characteristic Trx fold containing five β-strands surrounded by four α-helices, two cysteine residues in a highly conserved catalytic motif Trp-Cys-[Gly/Pro]-Pro-Cys, and the ability to reduce the disulfide bridge(s) of their target proteins (Katti, Lemasterf, & Eklund, 1990). In the small *Arabidopsis thaliana* genome, the thioredoxin family consists of complex isoforms, which are generally categorized by their primary sequence and subcellular localizations (Gelhaye, Rouhier, Navrot, & Jacquot, 2005). Among the six isoforms (TRX*f*, TRX*h*, TRX*m*, TRX*o*, TRX*x*, TRX*y*), the TRX*h*

belongs to a multigenic family of 8 different genes, which are differentially expressed (Reichheld, Mestres-Ortega, Laloi, & Meyer, 2002).

Upon sequence alignment of the eight *h*-type thioredoxins, AtTRXh3, AtTRXh4, and AtTRXh5 exclusively display a non-canonical active site motif WCPPC instead of the common WCGPC motif (Figure 3). And as previously mentioned, AtTRXh3 and AtTRXh5 have been identified to serve various roles in plant defense against pathogen infection (J. Lorang et al., 2012a; Sweat & Wolpert, 2007; Tada et al., 2008). In addition to their roles in NPR1 monomerization and induction of PR genes, AtTRXh3 and AtTRXh5 have implications in the JA pathway. For instance, AtTRXh5 is encoded by the *LIV1* (*locus of insensitivity to victorin1*) locus and confers sensitivity to victorin, a virulence effector that necrotrophic fungus *Cochliobolus victoriae* that causes Victoria blight releases for pathogenesis in host plants. The loss of AtTRXh5 function in mutant plants has been found to be partially rescued by AtTRXh3 (Sweat & Wolpert, 2007). Furthermore, expression of AtTRXh5 is not limited to during pathogen challenge but also induced by wounding, senescence, and abscission (Christophe Laloi, Mestres-ortega, Marco, Meyer, & Reichheld, 2004).

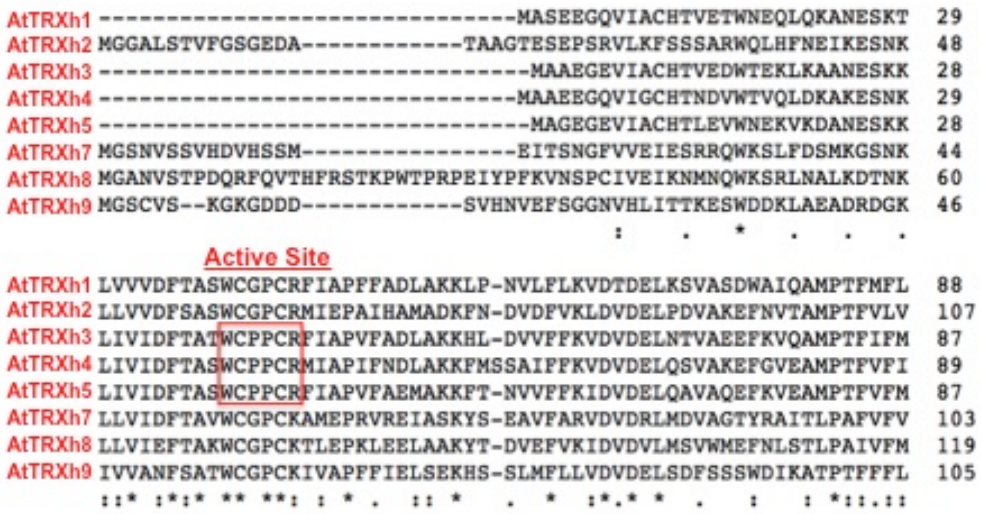


Figure 3. Amino acid sequence alignment of the cytosolic (*h*-type) thioredoxins in *A. thaliana.* The alignment was executed via ClustalW with the protein accession codes as follows: AtTRXh1: At3g51030; AtTRXh2: At5g39950; AtTRXh3: At5g42980; AtTRXh4: At1g19730; AtTRXh5: At1g45145; AtTRXh7: At1g59730; AtTRXh8: At1g69880; AtTRXh9: At3g08710. The conserved active site motif WCPPC is enclosed in red box. An * (asterisk) denotes positions which have a single, fully conserved residue; a : (colon) denotes conservation between groups of strongly similar properties – scoring > 0.5 in the Gonnet PAM 250 matrix; a . (period) denotes conservation between groups of weakly similar properties – scoring = < 0.5 in the Gonnet PAM 250 matrix.

Recently, AtTRXh5 has been reported to bind to the fungal toxin victorin and is guarded by *LOV1* (*locus orchestrating victorin effects1*), a coiled-coil NB-LRR *R* gene that confers susceptibility to *C. victoriae*. The activity of LOV1 also requires co-expression of AtTRXh5 and its binding to victorin, suggesting that *C. victoriae* achieves pathogenesis (Gilbert & Wolpert, 2013; J. M. Lorang, Sweat, & Wolpert, 2007; J. Lorang et al., 2012b) by targeted inhibition of a thioredoxin that plays important roles in defense (Gilbert & Wolpert, 2013; J. M. Lorang, Sweat, & Wolpert, 2007; J. Lorang et al., 2012).

Interplay of Phytohormones During Pathogen Challenge

In response to various pathogen challenges, interaction of 4 major plant hormones- salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA)- take place. Wide array of pathogens trigger an orchestration of different concentrations of SA or JA/ET in plants for mounting the appropriate defense pathway against a particular type of pathogen. For instance, necrotrophic pathogens and herbivores are sensitive to the plant's JA/ET-mediated defense pathway and biotrophic pathogens are sensitive to the SA pathway (Figure 4). A concept often labeled as the SA-JA/ET crosstalk, the induction of two groups of these phytohormones is regarded as mutually antagonistic- heightened resistance against necrotrophs rendered by the activation of JA pathway results in higher susceptibility to biotrophic pathogens, which also occurs interchangeably. SA-mediated defense pathway is characterized by expression of defense genes such as *PR-1* and subsequent mounting of SAR, while JA-pathway triggers synthesis of cell wall components and expression of JA-regulated defense genes such as *PDF1.2* and *COI1* (El Oirdi et al., 2011; Gimenez-Ibanez & Solano, 2013; Glazebrook, 2005; Pieterse, Van der Does, Zamioudis, Leon-Reyes, & Van Wees, 2012; Xie, 1998).

Although the antagonism between the SA and JA/ET-pathways are comprehensively documented, there have also been findings that suggest that the concept is perhaps overly simplified. For instance, an elevated SA level has been found to be necessary for plant defense against a necrotrophic pathogen, *Plectosphaerella cucumerina* (Berrocal-Lobo, Molina, & Solano, 2002). And global expression profiling in *Arabidopsis* has revealed a number of genes that were co-induced upon SA and JA

treatments, which suggests that the SA and JA/ET-pathways may have some overlapping members (Schenk et al., 2000). Therefore, the approach to dissecting the SA and JA/ET-dependent defense signaling pathways should include a complex perspective of biochemical network of regulatory interactions, rather than a simplified view of hormonal antagonisms.

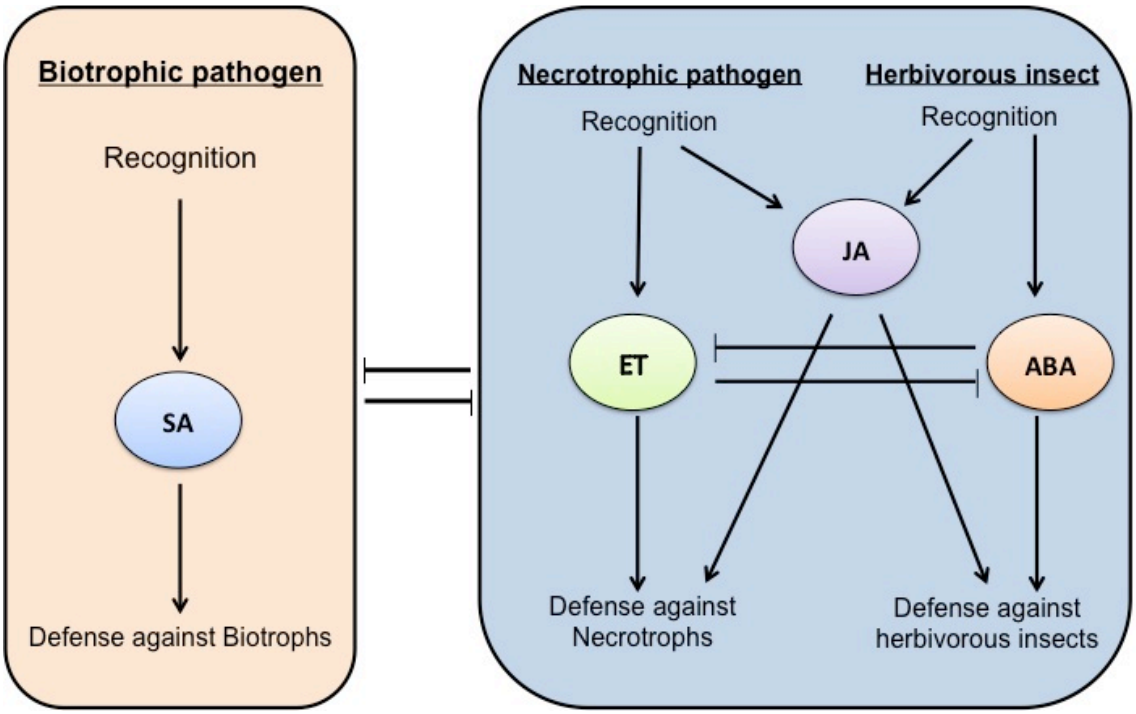


Figure 4. Interplay of phytohormones in immune signaling

AtTRXh3 and AtTRXh5 in Plant Defense Against Biotrophic and Necrotrophic Pathogens

Fluctuation in the cellular redox state plays crucial roles in regulating the plant defense pathways. Thioredoxins are important enzymes that mediate the activities of numerous proteins by using their highly reactive pair of cysteines in the active site to catalyze the thiol-disulfide switches (Buchanan & Balmer, 2005). A cytosolic

thioredoxin, AtTRXh5, regulates the activity of NPR1 by catalyzing its oligomer-to-monomer switch for activation of defense-related genes and mounting of SAR. Furthermore, both AtTRXh3 and AtTRXh5 are required for optimal induction of *PR1* gene upon induction with SA, which is an important transcriptional regulation event for defense against biotrophic pathogens. These two thioredoxins have also emerged as important members of victorin-mediated pathogenesis, suggesting that they are involved in eliciting defense response against necrotrophic pathogens (J. Lorang, 2012; Mpmi, Gilbert, & Wolpert, 2013; Sweat & Wolpert, 2007; Tada et al., 2008).

Despite the implications in the current literature regarding the importance of AtTRXh3 and AtTRXh5 in plant defense, a comprehensive resource for the targets of thioredoxins during plant defense is still incomplete. In this study, we analyzed the roles of AtTRXh3 and AtTRXh5 in defense against both biotrophic and necrotrophic pathogens. Infection bioassays were performed on *trxh3, trxh5*, and *trxh3trxh5* mutants with *Pseudomonas syringae* DC3000 and *Botrytis cinerea (B. cinerea)*. We observed that although AtTRXh5 is necessary for catalyzing the NPR1 oligomer-to-monomer switch, it is not required for induced resistance. Our bioassay data also suggests that reduction of NPR1 by AtTRXh5 may be replaced by other thioredoxins residing in the cytosol, thereby enabling plants to mount IR even in the absence of AtTRXh3 and AtTRXh5. In response to challenge by a necrotrophic fungal pathogen *B. cinerea*, we observed that both AtTRXh3 and AtTRXh5 are required for full immunity.

We also report a quantitative redoxome of proteins that were identified to be redox regulated upon defense elicitation by using an isotope coded affinity tag (ICAT)-based proteomic approach in wild-type, *trxh3*, *trxh5*, and *trxh3trxh5* mutant backgrounds.

From this experiment, we identified higher number of BTH-induced redox-sensitive proteins in trx mutants in comparison to wild type. And interestingly, majority of the defense-related proteins found to be redox-regulated was chloroplast-localized, suggesting that absence of AtTRXh3 and AtTRXh5 activates a stress response via heightened redox-regulation of chloroplastic proteins.

As an approach to further investigate the interactors of AtTRXh3 and AtTRXh5 during plant defense, we constructed mutant *trx* for an *in vivo* pull-down of thioredoxin interactors upon defense elicitation. Preliminary *trx* affinity pulldown in *Nicotiana benthamiana* proved a functional pulldown system of defense-related proteins that potentially interact with TRX during defense.

Results

Identification and Genetic Characterization of the *Attrxh3* and *Attrxh5* mutants

To gather genetic evidence for the investigation of the roles of thioredoxins in plant defense, we obtained knockout mutants from the Salk Institute T-DNA insertion collection (Alonso et al., 2003). Mutants with T-DNA insertion in the first intron of AtTRXh3 (SALK_111160) and in the first exon of AtTRXh5 (SALK_144259) were isolated through genotyping. To generate the double *trxh3trxh5* mutant, plants carrying homozygote insertions in AtTRXh3 and AtTRXh5 were cross-fertilized to obtain the F_1 progeny, which was self-fertilized. Genotyping of the F_2 progeny led to the isolation of homozygous mutants carrying both insertions in *AtTRXh3* and *AtTRXh5*. The absence of *AtTRXh3* and *AtTRXh5* mRNAs was confirmed through semi-quantitative RT-PCR analysis (Figure 5).

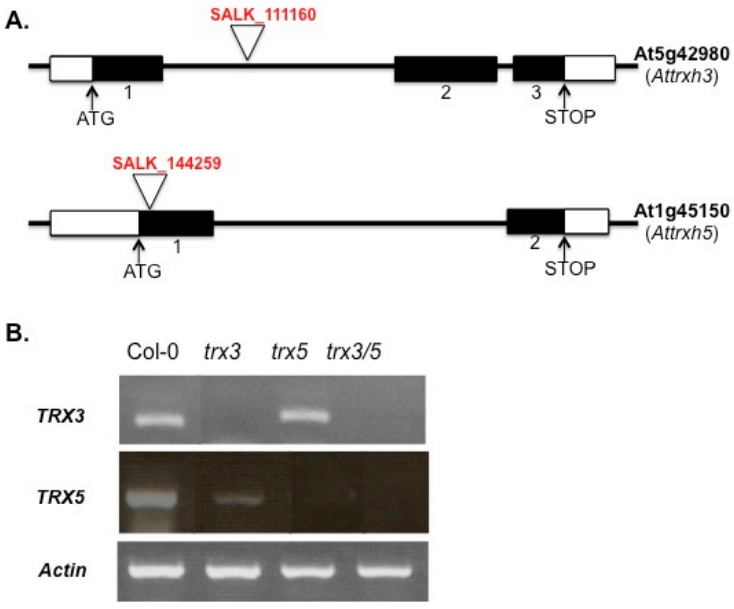


Figure 5. Isolation of SALK T-DNA insertion mutant lines (A) Schematic display of gene structures and T-DNA insertion locations of *Attrxh3* (SALK_111160) and *Attrxh5*

Figure 5. Isolation of SALK T-DNA, Continued. (SALK_144259). Exons are denoted as black boxes and introns as lines, and 5' and 3' untranslated regions are shown as white boxes. Triangles indicate sites of the T-DNA insertions. (B) Absence of *AtTRX3* and *AtTRX5* transcript levels in wild type, *trx3*, *trx5*, and *trx3/5* were confirmed via semi-quantitative RT-PCR using gene-specific primers. Actin demonstrates positive control for RT-PCR.

The homozygous *trxh3*, *trxh5*, and *trxh3trxh5* mutants displayed variations in sizes upon maturation. In comparison to wild type, *trxh3* displayed a dwarf phenotype, with shorter petioles and smaller leaves. On the other hand, *trxh5* was larger in size with longer petioles and bigger leaves than wild type. The double *trx* mutant displayed a growth phenotype similar to that of *trxh3* mutant- with shorter petioles and smaller leaves in comparison to wild type (Figure 6). These growth-related phenotypes exemplified by *trxh3* and *trxh5* mutants indicate an epistatic interaction between the two genes, as suggested by the *trxh3trxh5* mutant phenotype. Epistasis refers to a phenomenon during which interaction between different genes lead to a specific phenotype (Cordell, 2002). Our observation of the *trxh3trxh5* mutant phenotype suggests that epistatic interaction between *trxh3* and *trxh5* mediates the growth phenotype of the double mutant, in which *trxh3* masks the effect of *trxh5* to produce a phenotype characterized by smaller leaves and shorter petioles.

Figure 6. TRXh3 is epistatic to TRXh5. Picture shows growth phenotypes of 5-week-old *trx* mutants. In comparison to wild type, *trxh3* and *trxh3trxh5* mutants display smaller

Figure 6. TRXh3 is epistatic, Continued. leaves with shorter petioles; *trxh5* mutant displays larger leaves with longer petioles compared to wild type.

Absence of *AtTRXh3* and *AtTRXh5* does not inhibit induced resistance

As previously mentioned, both AtTRXh3 and AtTRXh5 have been shown to be required for full induction of *PR* genes and AtTRXh5 has been identified as the key reducing agent of NPR1, a master SAR-regulatory protein that is required for plant defense against biotrophic pathogens (Tada et al., 2008). Therefore, we hypothesized that these two thioredoxins are required, or can rescue each other's absence, for successfully mounting IR during defense against biotrophic pathogens. As an approach to answer this hypothesis, single and double *trx* mutant plants were subjected to *Pseudomonas syringae* DC3000 infection after induction of SAR by treatment with BTH. Quantitation of bacterial growth in the plants was performed 4 days post inoculation with *P. syringae.* As anticipated, wild type displayed decreased amount of bacterial growth when defense-induced by BTH treatment and the *npr1* mutant showed higher bacterial growth with impaired IR. Our data is also in line with what has been previously reported about single *trxh3* and *trxh5* mutants, which demonstrated slightly less bacterial growth than wild type and decreased susceptibility to *P. syringae* upon SAR induction. Mutant that lacks NADPH-dependent thioredoxin reductase A (*NTRA*), which replenishes the pool of reduced cytosolic thioredoxins, demonstrated impaired IR similarly to *npr1* mutant (C Laloi et al., 2001; Tada et al., 2008). Our data shows for the first time that absence of both *trxh3* and *trxh5* does not inhibit IR development during defense response (Figure 7). Plants lacking both *trxh3* and *trxh5* genes could still be primed by BTH for developing an increased resistance to *P. syringae* infection. Our observations led to two conclusions: (1)

that although the AtTRXh5 is necessary for catalyzing the NPR1 oligomer-to-monomer switch, it is not required for IR activation (2) function of AtTRXh5 in reduction of NPR1 can be replaced by other thioredoxins in the cytosol, thus enabling plants to mount IR even in the absence of AtTRXh3 and AtTRXh5.

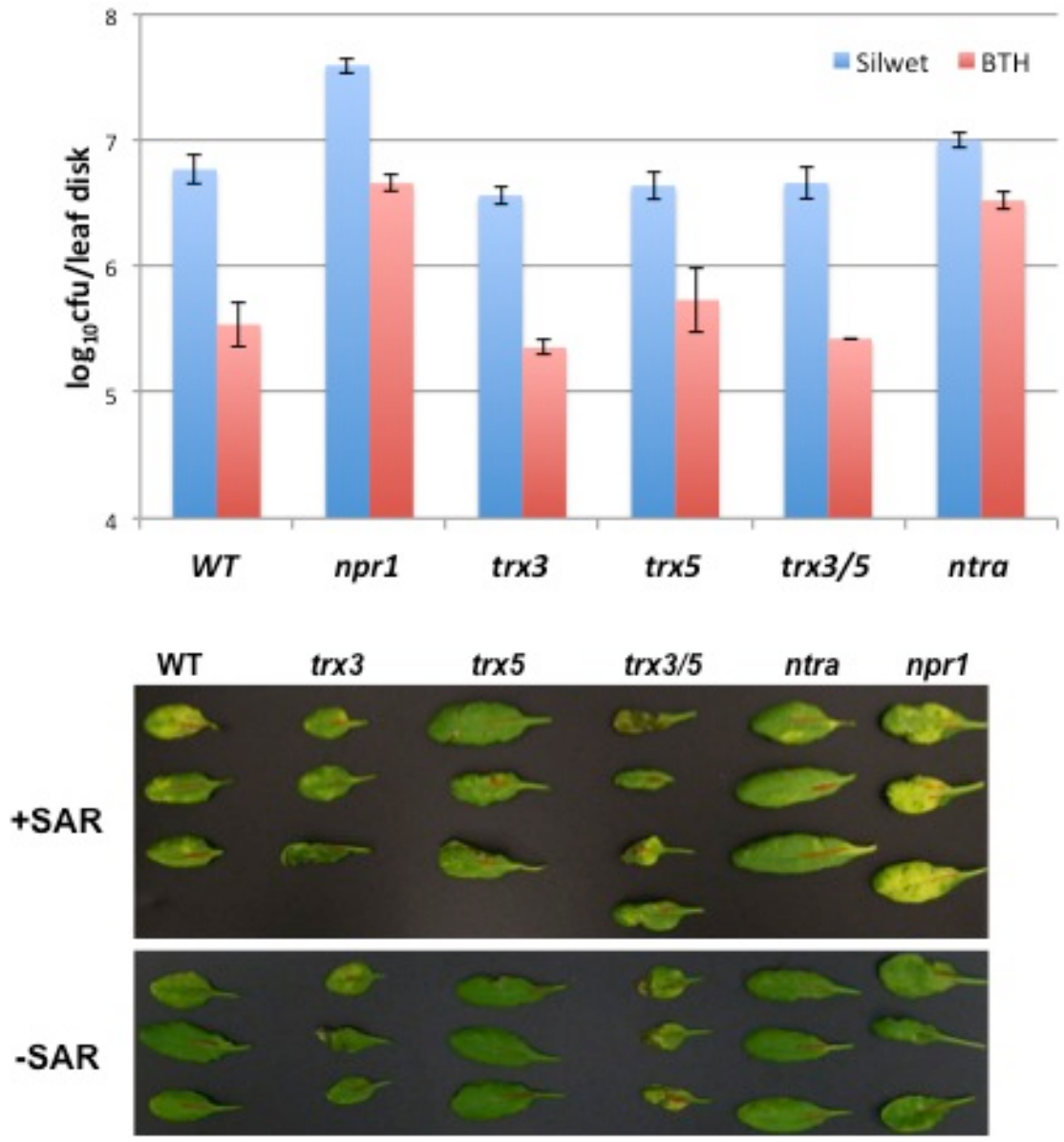


Figure 7. Quantitation of bacterial growth in *trx* mutants- 4 days post infection with *Pseudomonas syringae* DC3000. Blue bars signify quantitation of bacterial growth in plants that were not primed with BTH prior to infection (silwet); red bars indicate

Figure 7. Quantitation of bacterial growth, Continued. quantitation of bacterial growth in plants primed with BTH prior to infection (BTH). Data is shown as mean $\log_{10}$ colony-forming units (cfu) per cm^2 leaf tissue.

Thioredoxins are required for immunity against *Botrytis cinerea*

Previous reports have identified AtTRXh5 as a target of fungus *C. victoriae*, which secretes victorin that binds to AtTRXh5 for conferring susceptibility in plant host. Therefore, mutation in AtTRXh5 causes insensitivity to victorin (while AtTRXh3 can partially rescue this mutation) and thus resistance to the fungal infection (J. Lorang et al., 2012b; Sweat & Wolpert, 2007). Taken together what is currently understood regarding AtTRXh3 and AtTRXh5 as fungal targets for gaining virulence and their roles in the SA-mediated defense pathway, we had inquired whether these two thioredoxins serve importance in the JA-mediated defense pathway. As an approach to resolve our question, we challenged *trxh3*, *trxh5*, and *trxh3trxh5* to infection by a necrotrophic pathogen, *Botrytis cinerea*. Pathogenicity analysis was performed by drop inoculating detached leaves from 5-week-old *Arabidopsis* plants with grape isolate of *B. cinerea*. Susceptibility to the infection was analyzed at 96 hours post inoculation by measuring the necrotic lesions on the leaves. As displayed in Figure 8, the susceptibility of *trxh5* plants to *B. cinerea* was similar to that of wild type plants, whereas *trxh3* and *trxh3trxh5* plants showed enhanced susceptibility. The *trxh3* mutant was slightly more susceptible to the fungal infection than wild type. However, the *trxh3trxh5* mutant showed greater susceptibility to *B. cinerea* than the *trxh3* mutant and was also statistically significant in difference than wild type ($P < 0.05$). The controls of the experiment were served by the two phytoalexin deficient mutants, *pad2* and *pad3*, which are known to accumulate suboptimal levels of antimicrobial compound called phytoalexin camalexin upon

pathogen infection (Glazebrook et al., 1997). As anticipated, *pad2* and *pad3* showed enhanced susceptibility to *B. cinerea*, which is consistent to our current understanding of camalexin synthesis and JA signaling, which are crucial events required for resistance to *B. cinerea* (Figure 8).

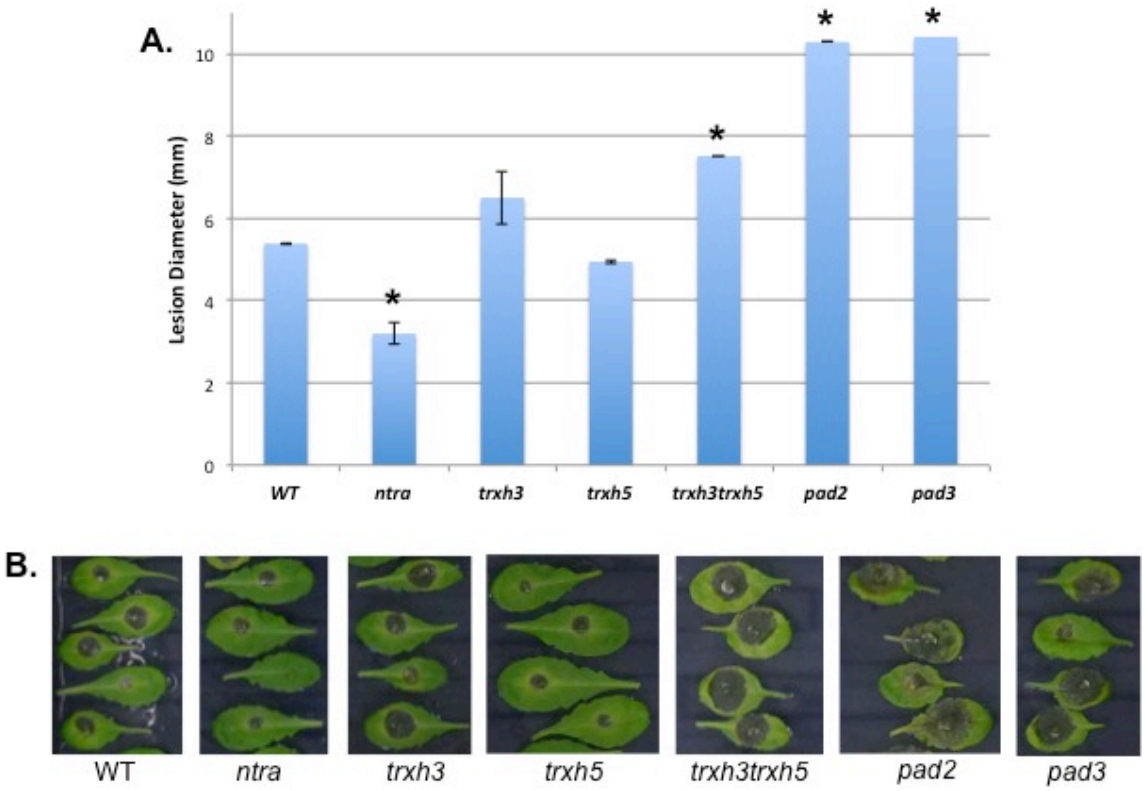


Figure 8. *trxh3* and *trxh3trxh5* are susceptible to *B. cinerea* infection. (A) Quantification of lesion sizes at 96 hours post inoculation. Error bars indicate the SD from 10 biological replicates. Data sets denoted with an asterisk are significantly different from wild type as calculated by Student's *t* test at $P < 0.05$. (B) Pictures of detached leaves from 5-week-old plants inoculated with *B. cinerea* grape isolate.

Interestingly, the *ntra* mutant displayed enhanced resistance to *B. cinerea* in comparison to wild type. This suggests that when reduced cytosolic thioredoxins are not replenished, there is an increased resistance to the necrotrophic infection. Furthermore, the *trxh5* mutant displayed similar disease susceptibility (if not slightly more resistant) to wild type. However, the enhanced susceptibility of the *trxh3trxh5* double mutant suggests

an epistatic relationship between the two genes. Given the loss of resistance to *B. cinerea* in *trxh3trxh5*, in which susceptibility phenotype of *trxh3* masks the *trxh5* phenotype; thus we determined that *trxh3* is epistatic to *trxh5*.

Induction of SA decreases susceptibility to *B. cinerea* in *Arabidopsis*

Although the general understanding of *Arabidopsis* defense against necrotrophic pathogens is predominantly JA-dependent, there have been reports that suggest this view as an oversimplification. For instance, exogenous application of JA did not enhance resistance to *B. cinerea* whereas BTH treatment induced resistance to the infection in tomato (Audenaert, Meyer, & Ho, 2002). And *Arabidopsis* grown in soil drenched with BTH prior to infection with *B. cinerea* demonstrated slower infection process, implying that defense against *B. cinerea* may be SA-dependent (Zimmerli, Métraux, & Mauch-Mani, 2001). Because we have so far observed AtTRXh3 and AtTRXh5 to serve significance in both SA and JA-mediated defense, we hypothesized that resistance against necrotrophic pathogen in *trxh3*, *trxh5*, or *trxh3trxh5* may be SA dependent. To test this hypothesis, we induced SA signaling in the mutant plants by treating them with BTH prior to *B. cinerea* infection. And interestingly, we observed that the primed wild type and mutant plants (except *npr1*) were less susceptible to *B. cinerea* infection than those that were not primed with BTH. Therefore, we were able to show that BTH, a synthetic analog of SA, protects *Arabidopsis* against gray mold fungus *B. cinerea*. Although disease progression was not halted, the degree of the disease symptoms was alleviated, as indicated by reduction in the lesion sizes post inoculation (Figure 9). On the contrary, *npr1* mutants were not defended against infection by the BTH treatment, which suggests

that NPR1 may be involved in the SA-mediated protection during defense against *B. cinerea*.

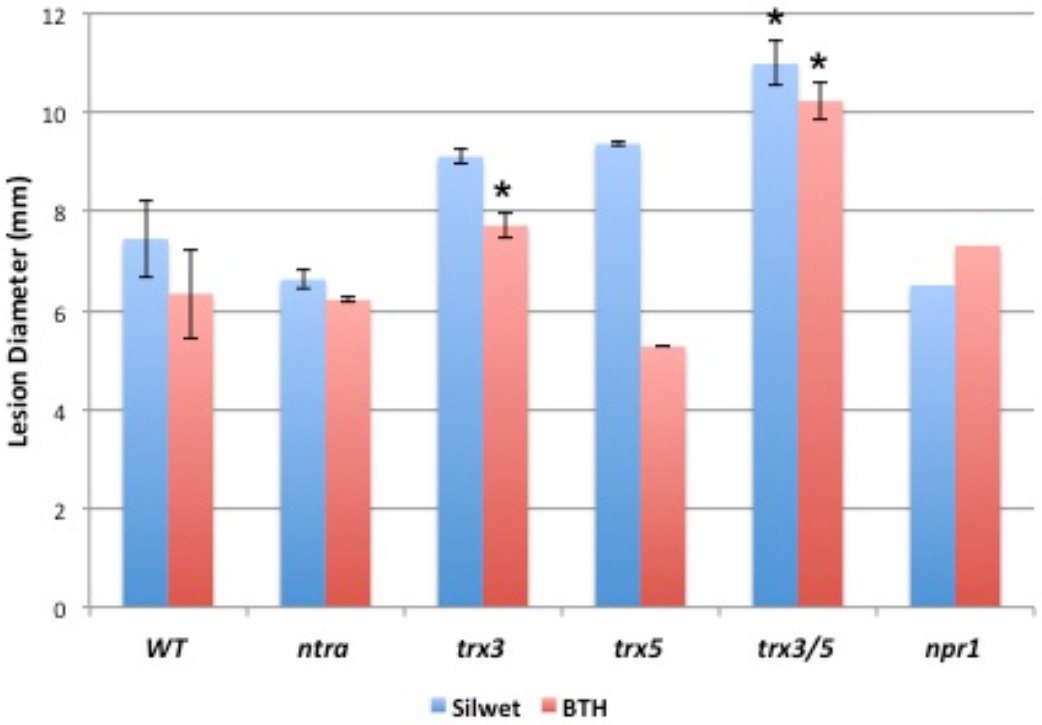


Figure 9. BTH protects plants against *B. cinerea* infection. Plants were pre-treated with 300 µM BTH 3-days prior to infection with *B. cinerea*. Quantification of susceptibility was obtained by measuring lesion sizes at 96 hours post inoculation using ImageJ software. Error bars indicate the SD from 10 biological replicates. Bars denoted with an asterisk display statistical significance in difference to wild type, which was calculated by Student's *t* test ($P < 0.05$).

Proteomics approach to identify potential TRX interactors during defense response

One of the recent advances in quantitative proteomics is the isotope coded affinity tag (ICAT) chemistry, which can be used in tandem with mass spectrometry for identification of proteins that undergo redox changes under stress conditions. ICAT reagent is a modified version of iodoacetamide (IAM), which is highly reactive to thiol

groups. The ICAT reagent is available in two isotopic versions- light ICAT reagent that has nine ^{12}C-atoms and heavy ICAT that consists of nine ^{13}C-atoms. Therefore the two forms of ICAT tag are chemically and physicochemically identical, except that they differ in mass by 9 Da (Figure 10a).

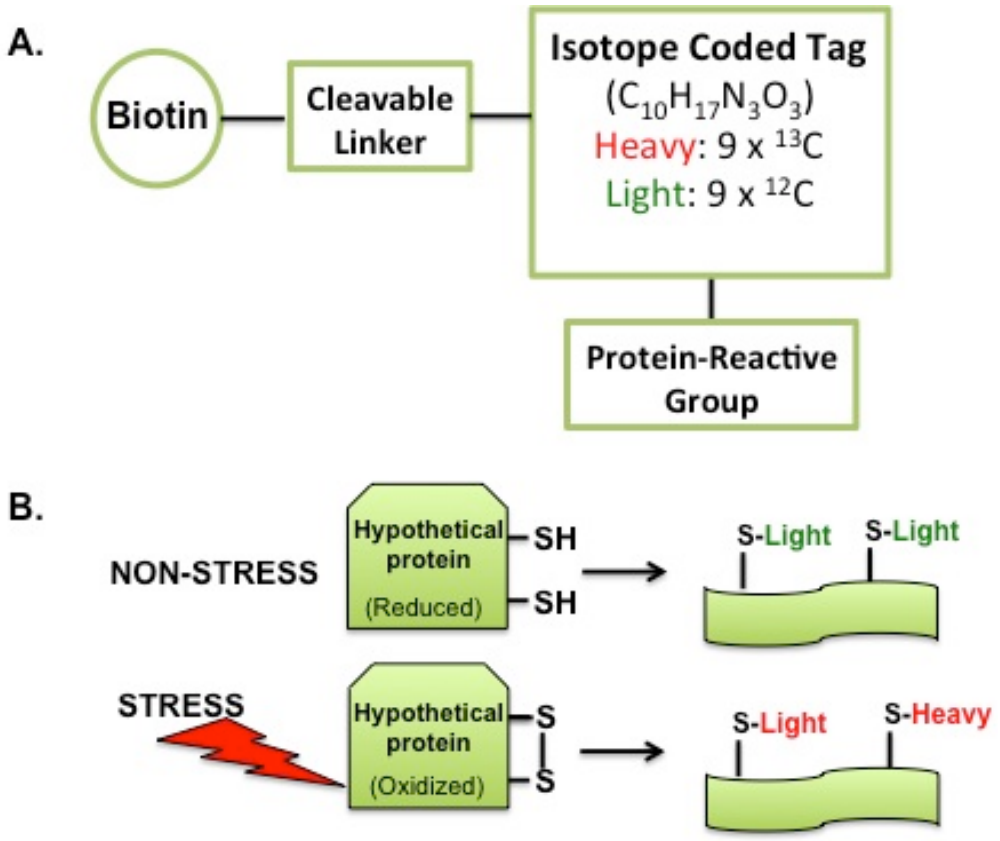


Figure 10. Identifying the redox state of proteins using ICAT approach (A) Schematic diagram displays chemical components of the ICAT reagent, an iodoacetamide derivative. (B) A hypothetical protein has reduced thiol groups under non-stress condition (upper) and has oxidized cysteines under stress condition (lower). Reduced cysteines are labeled with light ICAT and oxidized cysteines are labeled with heavy ICAT.

Leichert *et al*. group has recently developed a highly sensitive quantitative proteomics method for identifying redox-regulated proteins, which is comprised of both ICAT chemistry and differential thiol trapping to identify the oxidation state of various proteins. The technique also involves tagging the reduced and oxidized cysteines in a

cellular extract with light and heavy ICAT reagent, respectively (Figure 10b). Therefore, the ratios of reduced to oxidized cysteines can be obtained for hundreds of proteins from a single global experiment. Leichert *et al.* has developed and utilized this technique to identify proteins that show redox changes upon induction with oxidative stressors in *Escherichia coli* (Leichert et al., 2008; Lindemann & Leichert, 2012).

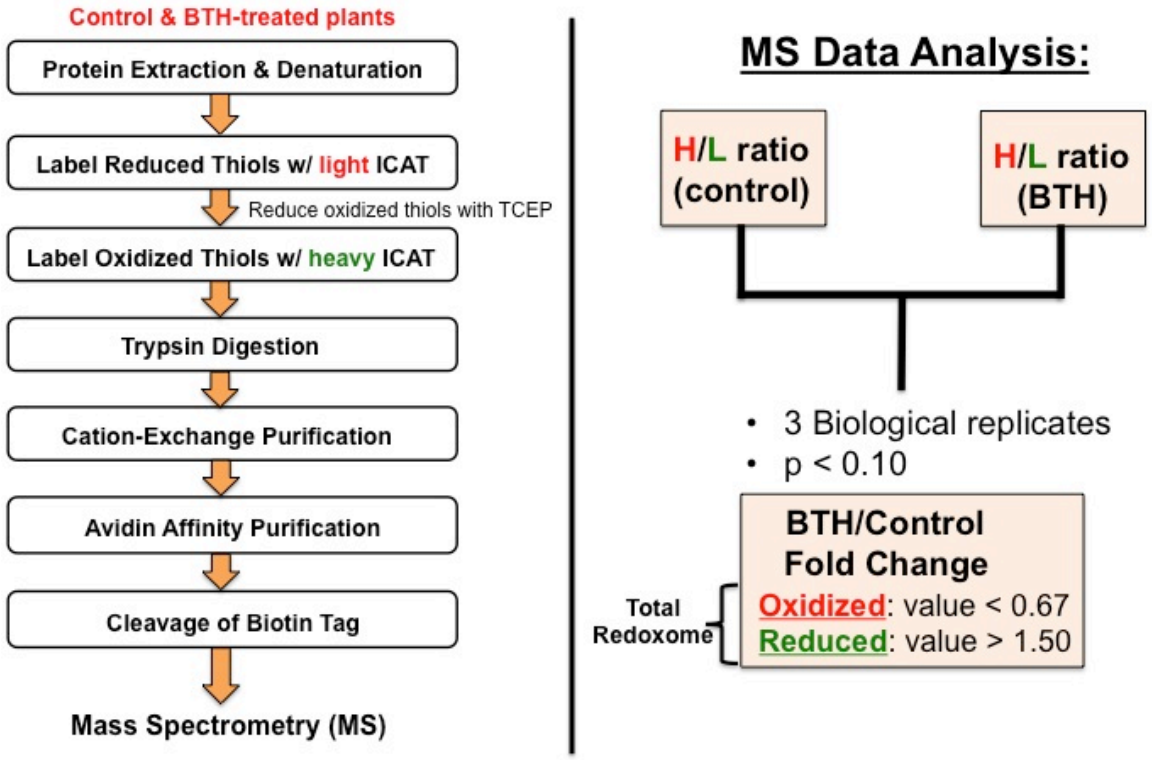


Figure 11. (Left) Proteomics workflow for identifying redox-regulated *Arabidopsis* proteins upon defense elicitation using ICAT. (Right) Summary of mass spectrometry (MS) data analysis

In our study, we have so far observed *trxh3*, *trxh5* and *trx3trxh5* mutants to display defense phenotypes upon infection with necrotrophic fungus, *B. cinerea*. And interestingly, the disease susceptibility was alleviated by BTH treatment, suggesting that SA-mediated protection against *B. cinerea* infection may be present. To further understand the significance of AtTRXh3 and AtTRXh5 in *Arabidopsis* defense against *B.*

cinerea and to build a comprehensive resource of proteins that are potentially redox-regulated by TRXs during defense, we applied the ICAT proteomic strategy to characterize the redoxomes of WT, *trxh3*, *trxh5* and *trxh3trxh5* mutants after defense elicitation. We activated defense in wild type and thioredoxin mutants by treating them with BTH for 2 hours and control plants with only silwet, which is a chemical surfactant that was included in the BTH mixture. After the 2-hour defense elicitation, the plants were harvested swiftly under dimly lit condition and flash frozen in liquid nitrogen for protein extraction. In order to achieve minimal oxygen conditions for thiol trapping, all buffers used for the protein extraction and first labeling step of tagging the reduced cysteines with light ICAT were purged with nitrogen gas. This step was very crucial to prevent any artifactual thiol disulfide exchange reactions and air-oxidation prior to the labeling steps.

For identification of proteins that change in redox status upon BTH treatment, mean heavy (H) to light (L) ratios of 3 biological replicates of each control and BTH-treated plants were calculated. Then the significance of differences between H/L ratios between silwet and BTH treatments was quantified by calculating the p-values, which was obtained by applying student's t-test on the ratios. Out of the proteins that made the p-value cutoff of $p<0.10$, the fold changes between the control and BTH-treated samples were determined by dividing the mean H/L ratio of BTH-treated samples by mean H/L ratio of control samples. The fold change cutoff for proteins was ≥ 1.5 for significant reduction and ≤ 0.67 for significant oxidation upon BTH treatment (Figure 11). Therefore the proteins that were identified after applying these cutoffs were considered as "total redoxome" of proteins that demonstrate change in redox state upon BTH treatment

(**Error! Reference source not found.**). This approach allowed us to identify proteins that contain highly reactive cysteine residues, whose thiol oxidation states may depend on AtTRXh3, AtTRXh5, or both, upon defense elicitation by BTH.

Table 1. Total redoxome of proteins from ICAT-labeling experiment. The table shows number of proteins that demonstrated change in redox state upon BTH treatment.

	Total Redoxome			
	Wild type	***trxh3***	***trxh5***	***trxh3trxh5***
Changes after BTH treatment	**11**	**24**	**10**	**22**
# of oxidized proteins after BTH	**8**	**21**	**6**	**14**
# of reduced proteins after BTH	**3**	**3**	**4**	**8**
Number of defense-related proteins	**4**	**14**	**5**	**11**

Table 2. Identification of defense-related redox-sensitive proteins by ICAT labeling method in wild type. Columns show protein identification, function, p-value ($p<0.1$), normalized expression ratio (heavy/light), and reduction or oxidation upon BTH treatment.

	PROTEIN	ACCESSION	GO ANNOTATION	P-VALUE	RATIO	RED/OX
WT	FERREDOXIN 2 (FED2)	AT1G60950	Encodes major leaf ferredoxin	0.08	0.242	Reduced
	AT4G29520	AT4G29520	Mutant demonstrates change under NaCl stress; expressional change during CaLCuV infection	0.003	0.001	Reduced
	SENESCENCE ASSOCIATED GENE 2 (SAG2)	AT5G60360	Senescence-associated thiol protease aleurain	0.052	0.0005	Reduced
	BETA-GLUCOSIDASE HOMOLOG (BGL1)	AT1G52400	ABA metabolic process, defense response to fungus, ER body organization	0.092	2.001	Oxidized

Table 3. Identification of defense-related redox-sensitive proteins by ICAT labeling method in *trxh3*, *trxh5*, and *trxh3trxh5* mutants.

	PROTEIN	ACCESSION	GO ANNOTATION	P-VALUE	RATIO	RED/OX
trxh3	PHOTOSYSTEM II (PSII)	AT1G51400	Response to UV-B, wounding, ozone	0.04	0.539	Reduced
	SENESCENCE ASSOCIATED GENE 2 (SAG2)	AT5G60360	Senescence-associated thiol protease aleurain	0.01	0.506	Reduced
	PHOSPHORIBULOKINASE (PRK)	AT1G32060	Protein binding, ATP binding, response to cold, bacterium, peptidyl-cysteine S-nitrosylation	0.03	0.415	Reduced
	KETOACYL-ACP SYNTHASE (KAS1)	AT5G46290	Fatty acid synthesis, chloroplast division & embryo development, response to cold	0.04	0.385	Reduced
	DEFENSIN-LIKE FAMILY PROTEIN (DEFL)	AT2G43530	Defense response, maltose metabolic process, starch biosynthetic process	0.10	0.102	Reduced
	CATALASE 3 (CAT3)	AT1G20620	Catalyzes H2O2 breakdown, lipid catabolism, response to nitrogen starvation and cold, regulation of defense response, photorespiration, systemic acquired resistance	0.09	0.0006	Reduced
	LYSYL-tRNA SYNTHETASE 1 (KRS1)	AT3G11710	ATP binding, cullin deneddylation, lysyl-tRNA aminoacylation, nucleotide biosynthesis	0.09	0.0006	Reduced
	SHORT-CHAIN DEHYDROGENASE 1 (SDR1)	AT3G61220	Defense response, flavonoid biosynthesis, oxidation-reduction, response to UV-B, karrikin, sucrose	0.09	0.0006	Reduced
	FRUCTOSE-BISPHOPHATE ALDOLASE 6 (FBA6)	AT2G36460	Glycolysis, response to cadmium ion and salt stress	0.09	0.0006	Reduced
	40S ribosomal protein	AT1G72370	Cell wall organization, response to cadmium ion, osmotic stress, salt stress; translation, water transport	0.002	0.0005	Reduced
	ANTHRANILATE SYNTHASE ALPHA SUBUNIT 1 (ASA1)	AT5G05730	MAPK cascade, ABA & SA signaling, defense response to bacterium, fungus, wounding; glucosinolate biosynthetic process,	0.001	0.0003	Reduced
	SEDOHEPTULOSE-BISPHOSPHATASE (SBPase)	AT3G55800	MAPK cascade, carbohydrate biosynthetic process; defense response to bacterium, fungus; JA & SA mediated signaling pathways; regulation of hypersensitive response; SA biosynthetic process	0.001	0.0003	Reduced
trxh5	MONODEHYDROASCORBATE REDUCTASE 6 (MDAR6)	AT1G63940	Protein glutathionylation, response to JA stimulus, response to symbiotic fungus, zinc ion, ozone	0.01	0.0007	Reduced
	CYCLASE FAMILY PROTEIN	AT4G34180	Golgi organization, glycolysis, hyperosmotic response, response to salt stress, water transport, cadmium ion, temperature stimulus	0.005	0.0004	Reduced
	SHORT-CHAIN DEHYDROGASE (SDR1)	AT3G61220	Defense response, flavonoid biosynthesis, oxidation-reduction, response to UV-B, karrikin, sucrose stimulus	0.08	0.0003	Reduced
	DEHYDROASCORBATE REDUCTASE (DHAR1)	AT1G19570	Protein glutathionylation, response to JA, ozone, symbiotic fungus, zinc ion	0.06	7.45	Oxidized
	CALRETICULIN 1B (CRT1B)	AT1G09210	Protein folding, response to ER stress, heat, oxidative stress, salt stress, systemic acquired resistance	0.08	3.46	Oxidized

Table 3, continued. Identification of defense-related redox-sensitive proteins by ICAT labeling method in *trxh3*, *trxh5*, and *trxh3trxh5* mutants.

	PROTEIN	ACCESSION	GO ANNOTATION	P-VALUE	RATIO	RED/OX
trxh3trxh5	LHCB1.1	AT1G29920	Photosynthesis, response to blue light, far red light, fructose stimulus, red light	0.03	0.65	Reduced
	JASMONATE RESPONSIVE 1 (JR1)	AT3G16470	Golgi organization, calcium ion transport, cysteine biosynthesis, response to cold, JA, salt stress, wounding	0.09	0.57	Reduced
	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (G3PDH)	AT1G13440	Golgi organization, defense response to bacterium, salt stress, temperature stimulus, cadmium ion, oxidative stress; glucosinolate biosynthesis,	0.09	0.40	Reduced
	TUBULIN BETA 8 (TUB8)	AT5G23860	GTP catabolism, cytoskeleton organization, gluconeogenesis, protein polymerization, response to salt stress	0.09	0.002	Reduced
	ABERRANT GROWTH AND DEATH 2 (AGD2)	AT4G33680	Ethylene biosynthesis, systemic acquired resistance, SA mediated signaling pathway	0.02	0.001	Reduced
	SUPPRESSOR OF THE G2 ALLELE OF SKP 1) (SGT1A)	AT4G23570	Response to auxin, defense response to fungus, ubiquitin-dependent protein catabolic process	0.00001	0.001	Reduced
	CHAPERONIN 20	AT5G20720	Chloroplast organization, cysteine biosynthesis, superoxide dismutase activity, response to cadmium ion, cold, salt stress	0.00001	0.001	Reduced
	GLUTAMATE-1-SEMIALDEHYDE-2,1-AMINOMUTASE (GSA1)	AT5G63570	Cell differentiation, chlorophyll, cysteine, and tetrapyrrole biosynthesis, response to light	0.0002	0.001	Reduced
	FERREDOXIN 2 (FED2)	AT1G60950	Encodes major leaf ferredoxin	0.08	0.001	Reduced
	ALLENE OXIDE SYNTHASE (AOS)	AT5G42650	MAPK cascade, defense response to bacterium & fungus, glucosinolate biosynthesis, regulation of hypersensitive response, response to chitin, fungus, JA, wounding; SA biosynthesis	0.01	1283.0	Oxidized
	EMBRYO DEFECTIVE 1467 (EMB1467)	AT5G37510	ATP synthesis coupled electron transport, cellular respiration, response to oxidative stress, photorespiration	0.04	4.17	Oxidized

Data analysis revealed that there were a lower number of BTH-induced redox sensitive proteins in wild type background than in the mutants. Out of the identified redox-sensitive proteins in wild type, only 4 proteins had defense related annotations (Table 2). One of these proteins is FD2 (At1g60950), which encodes a chloroplast-localized major leaf ferredoxin and is a homolog of PFLP in *Capsicum annuum*. PFLP has been implicated to target the apoplast for enhancing disease resistance in plants during harpin-induced ROS generation, hypersensitive response, and expression of *AtrbohD*, which is associated with resistance against bacterial attack (Huang et al., 2004; Lin et al., 2010). And interestingly, FD2 has been previously revealed as one of the putative AtTRXh3 targets in *Arabidopsis* leaves via TRX affinity chromatography

(Marchand et al., 2004). Another redox-sensitive protein identified in wild type is a β-glucosidase called BGL1 (At1g52400), which is induced in leaves upon diamondback moth feeding and methyl-JA treatment (Stotz et al., 2000).

Chloroplast-localized and stress-responsive proteins upregulated in *trx* mutants

In comparison to wild type, more chloroplast-localized proteins were observed to be redox sensitive in the *trxh3*, *trxh5*, and *trxh3trxh5* mutants upon defense elicitation. The double mutant demonstrated the highest amount of chloroplast-localized proteins upon defense activation, with about 72.7% of identified proteins categorized as chloroplast-localized proteins. Additionally, all *trx* mutants (*trxh3, trxh5, trxh3trxh5*) displayed higher percentage of redox-sensitive proteins related to abiotic/biotic stress in comparison to wild type (Figure 12). In the *trxh3* mutant, 7 out of 14 defense-related proteins were chloroplast-localized proteins: PSII (At1g51400), PRK (At1g32060), KAS1 (At5g46290), CAT3 (At1g20620), RP40 (At1g72370), ASA1 (At5g05730), and SBPase (At3g55800). We observed only 5 defense-related proteins to show redox-sensitivity to BTH treatment in *trxh5* background, 3 of which were chloroplast-localized proteins (MDAR6 (At1g63940), DHAR5 (At1g19570), CRT1B (At1g09210)). And in the double mutant, we observed 11 defense-related proteins, majority of which were also chloroplast-localized (LHCB1.1 (At1g29920), JR1 (At3g16470), G3PDH (At1g13440), AGD2 (At4g33680), CPN21 (At5g20720), GSA1 (At5g63570), FD2 (At1g60950), AOS (At5g42650), EMB1467 (At5g37510)) (Table 3).

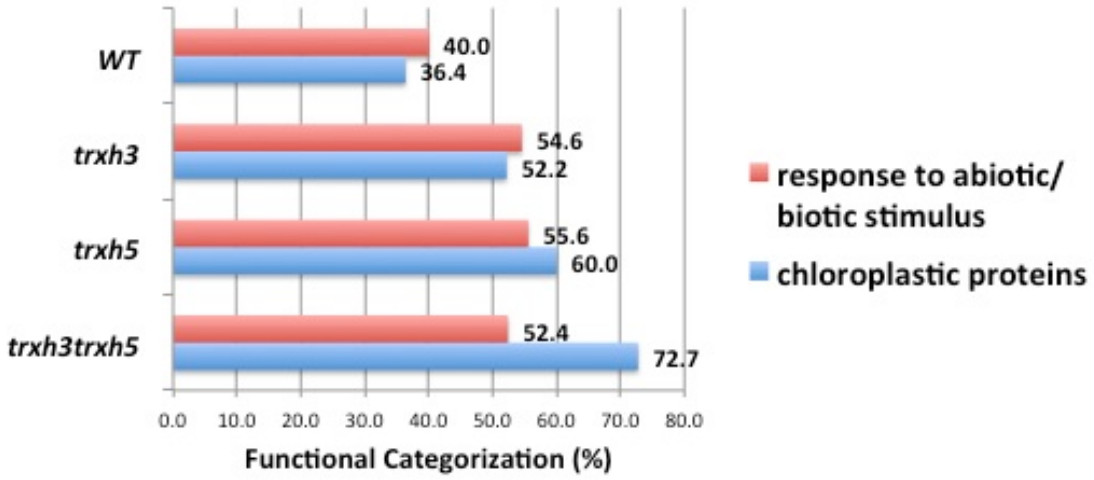


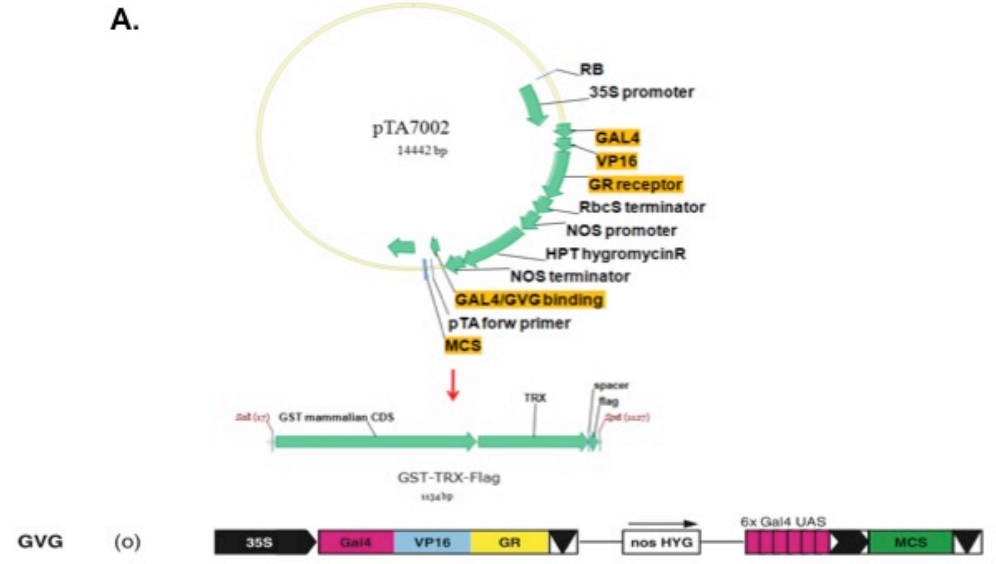
Figure 12. Comparison of the functional categorization of redox-sensitive proteins between wild type and *trx* mutants. Functional categorization was obtained by using the Gene Ontology (GO) annotation tool provided by TAIR.

Construction of mutant thioredoxins for *in vivo* TRX affinity pulldown

Through the ICAT labeling approach, we have identified proteins in wild type and *trx* mutant backgrounds that are both involved in plant immunity and redox-regulated. However, we aimed to further characterize the role of AtTRXh3 and AtTRXh5 in plant defense by identifying which of the redox-regulated proteins interacts with thioredoxins. Therefore, we generated transgenic *Arabidopsis* for trx affinity pull-down of TRX interactors after defense elicitation. To generate mutant *TRXh3C39S* and *TRXh5C39S*, one of the two active site cysteines of *TRXh3* and *TRXh5* was mutated to serine and cloned into a dexamethasone-inducible plant expression vector pTA7002 (Figure 13a).

Thioredoxins interact with their target proteins by catalyzing the thiol-disulfide exchange reaction. As conceptualized in Figure 13b, TRX normally interacts with its target protein by forming an intermediate mixed-disulfide bridge at the first reactive cysteine. Then the second cysteine of TRX reduces the intermolecular disulfide bridge,

resulting in target protein reduction and oxidation of TRX. However, in the mutant trx background, in which the first cysteine is mutated to a serine, the mixed disulfide bond cannot be reduced to facilitate release of the target protein. Therefore the mutant trx provides a system through which we can capture the TRX interactors (Hisabori et al., 2005).



(Hisabori et al., 2005)

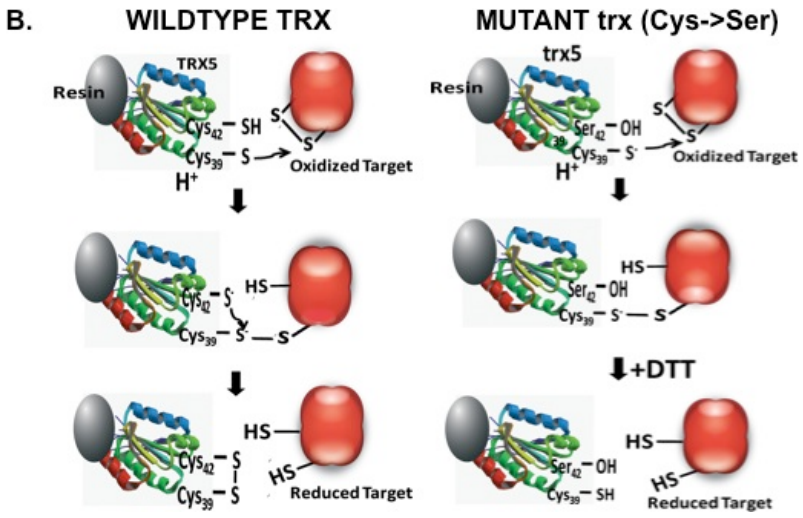


Figure 13. *In vivo* trx affinity pull-down system. (A) Diagram of dexamethasone-inducible vector pTA7002. Dexamethasone (DEX) binds to the glucocorticoid receptor

Figure 13. *In vivo* trx affinity, Continued. (GR) to cause nuclear localization. The Gal4 DNA binding domain then binds the 6X Gal4 upstream activation site and VP16 activation domain activates the transcription of the TRX gene. (B) Mutant trx containing Cys→Ser mutation selectively retains the mixed disulfide bond with its target protein, allowing the capture of target protein. Increased binding to the column reveals an increase in oxidation.

To analyze the integrity of transgene expression *in planta*, we performed a transient expression assay in tobacco (*Nicotiana benthamiana*). *Agrobacterium tumefaciens* was transformed with the *35S::TRXh3C39S-FLAG* and *35S::TRXh5C39S-FLAG* constructs and infiltrated into the leaf cells of *N. benthamiana* to deliver the transgenes. The agroinfiltrated leaves were then treated with dexamethasone for induction of transgene expression, and protein expression was analyzed via immunodetection (Figure 14).

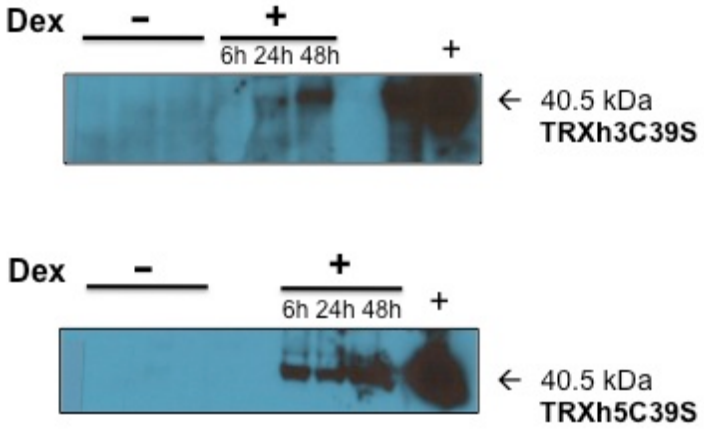


Figure 14. DEX-induced transient expression of mutant TRXh3C39S and TRXh5C39S in N. benthamiana. Western blot detection of FLAG-tagged mutant trxh3 and trxh5 displayed at 40.5 kDa. FLAG antibody used for immunodetection of trx proteins. Transient expression of TRXh3C39S and TRXh5C39S was detected 6 hours to 48 hours post DEX treatment.

***In vivo* pulldown of redox-regulated TRX interactors during defense response**

To confirm whether the pull-down of potential TRX interactors is functional *in planta*, we performed a preliminary pull-down in *N. benthamiana* using pTA7002

construct containing *TRXh5C39S*. Tobacco leaves were infiltrated with transformed *Agrobacteria* carrying the transgene and treated with BTH for defense activation. Expression of the transgenes was induced with DEX treatment for 24 hours to stabilize the transient interaction between TRXh5C39S and its target protein during defense activation. After protein extraction, cellular lysate was subjected to immunoprecipitation with FLAG antibody immobilized on agarose beads. The interactors of TRX from the pull-down were identified via mass spectrometry analysis (Table 4).

Table 4. Potential TRX interactors identified from *in vivo* TRX affinity pull-down in *N. Benthamiana* upon defense elicitation.

PROTEIN	ACCESSION ID	GO ANNOTATION	RATIO	TOTAL SPECTRA
Catalase 3 (CAT3)	At1G20620	Hydrogen peroxide catabolism, regulation of defense response, response to cold, systemic acquired resistance	1.02	32
RUBISCO ACTIVASE (RCA)	AT2G39730	MAPK cascade, defense response to bacterium, fungus; response to JA stimulus; SA-mediated signaling pathway	2.02	52
HSP70-2	AT5G02490	Response to bacterium, heat, hydrogen peroxide; SA biosynthetic process, systemic acquired resistance	1.83	88
GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C-2 (GAPC2)	AT1G13440	Defense response to bacterium, glucosinolate biosynthetic process, oxidation-reduction process, response to oxidative stress, salt stress	1.52	74
S-ADENOSYLMETHIONINE SYNTHETASE 1 (SAM1)	AT1G02500	Ethylene biosynthetic process, response to salt stress; response to wounding	1.69	32
LESION INITIATION 1 (LEN1)	AT1G55490	Beta subunit of the chloroplast chaperonin 60; mutants develop lesions on its leaves, expresses SAR and accelerated cell death	2.31	42

The preliminary pulldown in *N. benthamiana* proved a functional pulldown system of defense-related proteins that potentially interact with TRX. We observed more than 500 proteins in our mass spectrometry data that was pulled down using *35S::TRXh5C39S-FLAG.* Proteins that demonstrated significant change in abundance upon BTH treatment were filtered by applying the fold change cutoff of >1.5 and <0.67 to the BTH/control ratios. And from these candidate proteins, we observed about 12

proteins that were defense-related, some of which are shown in **Error! Reference source not found.**. One of these proteins, Catalase 3 (CAT3, At1g20620), is a defense–related protein that we have also detected from our ICAT labeling experiment. A member of the small multigene family of catalases in *Arabidopsis*, CAT3 is one of the key enzymes that metabolize hydrogen peroxide, which is one of the reactive oxygen species (ROS). And hydrogen peroxide (H_2O_2) is an important signaling molecule that is known to induce hypersensitive cell death in response to pathogen infection in plants (Du, Wang, Chen, & Song, 2008).

Discussion

This study involves an investigation of two cytosolic thioredoxins, AtTRXh3 and AtTRXh5, in their roles in defense against both biotrophic and necrotrophic pathogens. Although AtTRXh5 is well defined to be the key reducing agents of a master SAR-regulatory protein NPR1, we found through our bioassay experiments that they are not required for plants to mount induced resistance upon pathogen challenge. We also found that thioredoxins are required for full immunity against necrotrophic pathogen, possibly through an SA-mediated defense pathway. Finally, we utilized a proteomic method of ICAT-labeling to establish a resource of redox-regulated proteins involved in plant defense.

Thioredoxin h3 and h5 are not required for induced resistance

While AtTRXh3 and AtTRXh5 have been found to interact with NPR1, AtTRXh5 has been identified as the key reductant for NPR1 oligomer-to-monomer switch. Reduction of NPR1, a master SAR-regulatory protein, is necessary for the monomeric NPR1 to localize to the nucleus for transcriptional activation of *PR* genes. Expression of *PR* genes is necessary for mounting SAR, a phenomenon during which plants gain systemic secondary resistance against a wide array of pathogens (Tada et al., 2008). However, our bioassay data has revealed that the two cytosolic thioredoxins are not required for plants to mount induced resistance. We observed that the *trxh3*, *trxh5*, and *trxh3trxh5* mutants exemplified the ability to develop IR, as they showed greater

resistance to *Pseudomonas syringae* infection when primed with BTH treatment (Figure 7).

Our bioassay data also shows that mutant lacking NADPH-dependent thioredoxin reductase (NTRA), which is an enzyme that is responsible for reducing cytosolic thioredoxins and thus have replenished pool of reduced thioredoxins, does not have the ability to mount IR. The *ntra* mutant displayed impaired IR upon BTH treatment in a similar manner to *npr1* mutant (Figure 7). From the observations that absence of both AtTRXh3 and AtTRXh5 does not abolish IR, while impairment of replenishing reduced cytosolic TRXs inhibits IR, we were able to conclude that there may be other cytosolic thioredoxins that are required IR in plant defense against biotrophic pathogens.

AtTRXh3 and AtTRXh3 are required for immunity against *B. cinerea* through an SA-mediated pathway

Recent reports that have identified AtTRXh5 (and partially AtTRXh3) as a target of fungal toxin victorin secreted by *Cochliobolus victoriae*. Victorin is an effector secreted by the fungus that has been found to bind to thioredoxins for conferring sensitivity to its presence and thus susceptibility to the fungal infection. Furthermore, AtTRXh5 is encoded by the (locus of insensitivity to victorin1) LIV1 locus and is necessary for conferring sensitivity to victorin while overexpression of AtTRXh3 can partially compensate for the loss of AtTRXh5 (J. Lorang et al., 2012; Sweat & Wolpert, 2007). These findings have sparked our interest to investigate whether AtTRXh3 and AtTRXh5 have roles in the JA-mediated defense pathway. Indeed, our *Botrytis cinerea* infection bioassay data revealed that AtTRXh3 and AtTRXh5 are required for full

defense against the necrotrophic pathogen. Based on susceptibility quantification by measuring lesions that formed on the leaves after infection, the *trxh3* mutant showed greater average lesion size in comparison to wild type. The double *trxh3trxh5* mutant also displayed susceptibility to the fungal infection, suggesting that the defense phenotype of *trxh3* is epistatic to *trxh5*. The *trxh5* mutant did not show significant susceptibility to *B. cinerea*, but when both AtTRXh3 and AtTRXh5 were absent, the susceptibility phenotype of *trxh3* masked the phenotype of *trxh5* to result in susceptibility (Figure 8).

Our investigation of thioredoxins in defense against *B. cinerea* included a BTH-priming test, which has led to interesting observations. We observed that when the wild type, *trxh3, trxh5*, and *trxh3trxh5* plants were pre-treated with BTH prior to *B. cinerea* infection, they demonstrated enhanced resistance to the fungal infection than the control plants. The *npr1* mutant, on the contrary, did not demonstrate this priming effect. Therefore, we have concluded that the role of AtTRXh3 in defense against necrotrophic pathogen may be SA-dependent, as induction of SA resulted in enhanced resistance against *B. cinerea* infection (Figure 9). Our observations are in line with previous reports that show that BTH treatment causes WT plants to become more resistant than control plants in tomato and *Arabidopsis* grown in a BTH-drenched soil display slower infection process by *B. cinerea* (Audenaert et al., 2002; Ferrari, Plotnikova, De Lorenzo, & Ausubel, 2003; Zimmerli et al., 2001).

BTH treatment, or induction of SA, is known to activate the SAR signal transduction pathway. This pathway is generally understood as being activated in response to defense against biotrophic pathogens and is also antagonized by another defense-associated hormone, JA. Despite the widely accepted SA-JA/ET hormone

crosstalk in plant defense, there have been many implications that the antagonistic relationship is not always clearly observed (Berrocal-Lobo et al., 2002; Schenk et al., 2000). The results of our study correlates to the general idea that the SA and JA/ET pathways are not strictly antagonizing or isolated, as we've observed that AtTRXh3 and AtTRXh5 play importance in defense against both necrotrophic and biotrophic pathogens.

BTH causes more proteins to change in redox level in *trx* mutants than in WT

We utilized an ICAT-labeling approach to identify redox-regulated proteins during defense in WT and *trx* mutant backgrounds. Through this experiment, we aimed to identify which defense-related proteins are redox-regulated and also show dependence on AtTRXh3, AtTRXh5, or both, for proper redox-regulation. Upon mass spectrometry analysis, we obtained a total redoxome of proteins that demonstrated change in redox state upon defense activation (Table 1). And interestingly, in the trx mutants, we detected more proteins to change in redox level upon BTH treatment in comparison to wild type. The common trend in the redoxome of mutant *trxs* was that most of the proteins were in oxidized state upon BTH induction. This observation correlates with the idea that when we mutate thioredoxins, which are known to have reductive abilities, we would observe more proteins that are oxidized rather than reduced. Furthermore, in the *trx* mutants (*trxh3, trxh5, trxh3trxh5*), many of the BTH-induced redox-sensitive proteins were found to be chloroplast-localized proteins (Figure 12). Observation of an enrichment of chloroplast-localized proteins in the mutant plants was interesting because although the

chloroplast is best known as the location of photosynthesis, it is also a site of synthesis of plant hormones and lipids involved in defense signaling.

Possible Mechanism

In the possible mechanism through which thioredoxins serve importance in defense signaling, the thioredoxin regulates a protein with inhibitory function. As shown in Figure 15, there's a hypothetical redox-regulated protein that inhibits the JA-mediated defense pathway, thereby causing susceptibility to necrotrophic invasion. And normally, a thioredoxin changes the activity of this protein to relieve its inhibition of the JA-mediated pathway. Therefore if this thioredoxin is absent, the hypothetical protein retains its role of inhibiting the JA pathway, causing susceptibility to necrotrophic challenge.

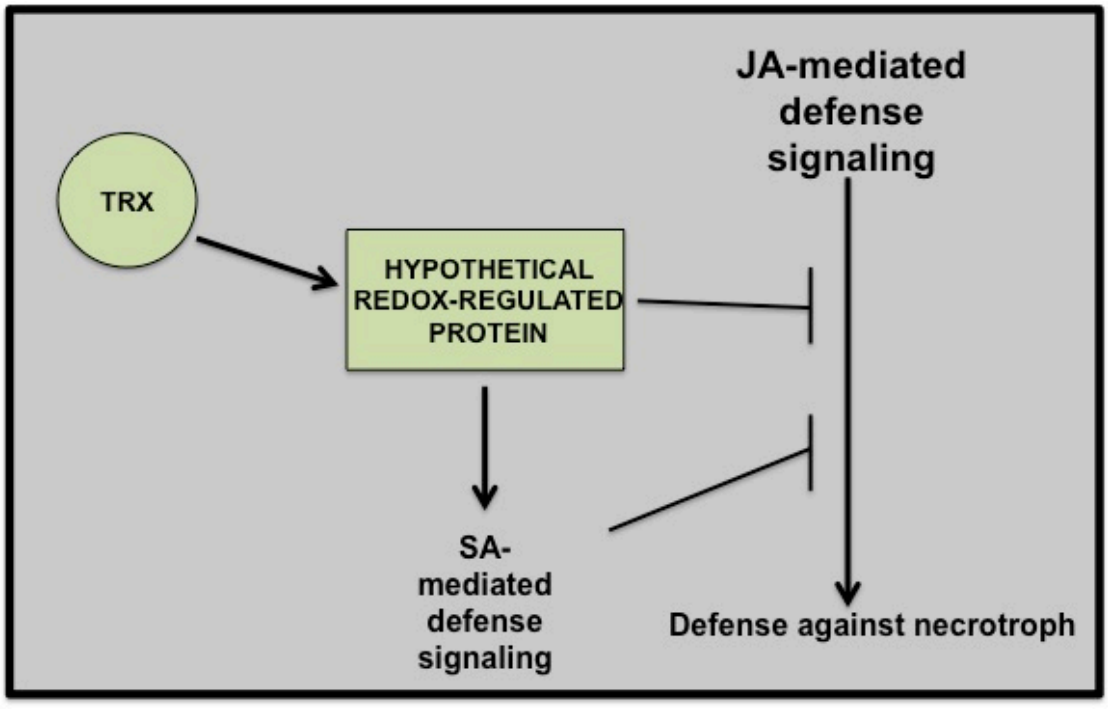


Figure 15. Possible mechanism through which thioredoxins regulate defense-related proteins.

This is a possible mechanism that could explain our observations from the *B. cinerea* infection bioassay, in which the *trx* mutants showed enhanced susceptibility to the infection. Furthermore, from our ICAT data, we identified several BTH-induced redox-sensitive proteins that are known to play roles in JA-mediated defense signaling. Therefore, based on this potential mechanism, we can utilize our ICAT data to select candidate proteins and subject them to mutational analysis. We could test whether the absence of these candidate TRX interactors leads to resistance or susceptibility to pathogen infection and confirm whether their activities depend on the reductive regulation by thioredoxins. These further analyses would greatly solidify our understanding of the specific proteins that depend on thioredoxins for their redox-regulation during plant defense.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotype Columbia seeds were vernalized at 4^{o}C for 2 d and then sown in pots containing a mixture of soil and vermiculite (3:1, v/v). Plants were grown in a controlled environment room at 22^{o}C constant temperature under a 16-h-light, 8-h-dark photoperiod.

T-DNA Insertion Lines and Mutant Cross

Seeds for T-DNA insertion mutants for *trxh3* (At5g42980; SALK 111160), *trxh5* (At1g45150; SALK 144259), *npr1-1* (At1g64280), and *ntra* (At2g17420; SALK 039152) were obtained from the ABRC. Genotypes of the *trx* mutants were confirmed by means of PCR analysis using gene-specific and T-DNA-specific primers. These were as follows: for *TRX3* (forward 5'-GCTGCGAGTAATCAAGTTTGC-3' and reverse 5'-ACCGACACAGAGACGAAGAAG-3'), *TRX5* (forward 5'-GTGGTGAATGATGTTTGTGTTCTGATTTG-3' and reverse 5'-CGTGTTTAAAACCAGTCTTAATGTGTGTCGC-3'), and T-DNA insertion specific Lba1 (5'-TGGTTCACGTAGTGGGCCATCG-3').

For generation of *trx3/trx5* double mutant, SALK 111160 was cross-fertilized with SALK 144259. The F1 plants were selfed, and the F_2 plants were screened for *trx3/5* genotype by PCR analysis using gene-specific primers as described above.

Mutagenesis of Active-Site Cys Residues

Thioredoxins mutated in their active site were obtained by site-directed mutagenesis by overlap extension using polymerase chain reaction (PCR) method. We used oligonucleotides designed to hybridize on the active center: trx3CS_F, trx3CS_R, trx3CSint_F, trx3CSint_R for *AtTRXh3*, trx4CS_F, trx4CS_R, trx4CSint_F, trx4CSint_R for *AtTRXh4*, trx5CS_F, trx5CS_R, trx5CSint_F, trx5CSint_R for *AtTRXh5*.

Plasmid Construction and Plant Transformation

The Dexamethasone-inducible pTA7002 plasmids carrying *trx3-FLAG*, *trx4-FLAG*, *trx5-FLAG* were electroporated into *Agrobacterium tumefaciens*, and the resulting bacteria were used to transform *trx3, trx4,* and *trx5* mutants by floral dip method. Transformants were selected on plates of Murashige and Skoog (MS) medium containing hygromycin B (15 μg/ml). Nontransformants develop chlorotic cotyledons and arrest at this developmental stage, while transformants containing the pTA7002 plasmids develop normally, with green cotyledons and leaves.

Protein analysis/Immunodetection

For the analysis of protein accumulation in Arabidopsis plants, leaf samples were flash frozen in liquid nitrogen and mechanically ground in equal volume of plant extraction buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100 and 0.1% SDS) supplemented with a 1:100 dilution of protease inhibitor cocktail (Roche). Protein extract was centrifuged for 5 minutes at 10,000 x g at 4^{o}C. The

supernatants were collected, protein concentration was measured and the protein extracts were boiled on SDS-loading buffer (125 mM Tris-HCl, 25% Glycerol, 5% SDS, 5% βME and 1% Bromophenol blue) and separated on 4-8% SDS polyacrylamide gel (SDS-PAGE). The proteins were then blotted onto a membrane using a Semi-Dry Transfer unit. Membranes were first blocked with a 5% milk-Phosphate Buffer Saline-Tween 20 (PBST) solution and then incubated in a 1% milk-PBST solution containing a 1:1000 dilution of anti-FLAG monoclonal antibody (Sigma). Protein complexes were labeled with the appropriate horseradish peroxidase-conjugated secondary antibody and detected using the Enhanced Chemiluminescence Reagent (ECL).

Chemical Treatments

Dexamethasone (DEX), a glucocorticoid derivative, was purchased from (brand). DEX was reconstituted in 50:50 100% ethanol and DMSO before use. For DEX treatment, 6-week-old plants were sprayed with a 50-μM solution containing 0.01% (*w/v*) silwet.

For induction of defense responses, two- to four-week-old plants were sprayed with 300 μM benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) supplemented with 0.01% Silwet.

RNA Isolation and RT-PCR

RNA was isolated from frozen tissue samples by Qiagen RNA isolation kit. cDNA was produced by first strand synthesis using oligo(dT) primer and SuperScript Reverse Transcriptase (Invitrogen, Carlsbad, CA).

Pathogen Infection

3 to 4-week-old *Arabidopsis* plants were sprayed with 0.05% silwet (control) or 300 μM BTH (defense activation). The primed plants were infected three days later with the virulent strain of *Pseudomonas syringae* DC3000 at OD_{600} = 0.0002. Tissue samples (0.5 cm^2 leaf discs) were harvested from inoculated leaves at 0, 2 and 4 days after injection. Leaf discs were homogenized in 10 mM $MgSO_4$ and quantified by plating appropriate dilutions on King's B agar containing rifampicin (50 mg ml^{-1})

For *Botrytis cinerea* infection, leaves were excised from 3 to 4-week-old plants and placed on 0.5% plant agar in plastic trays. Five microliters of the 50,000 spores/ml grape isolate spore suspension was drop inoculated onto the middle of the leaves. High humidity was maintained by covering the plastic trays with clear plastic lids and placed at room temperature. For analysis of disease susceptibility, the diameter of the lesions produced was measured at 48, 72, and 96-hour post-inoculation using ImageJ software.

Protein Extraction from *Arabidopsis*

Frozen tissue samples were homogenized in the Oscillating Mill MM400 (Retsch®) after chilling in liquid nitrogen. Resulting powder was stirred into 1.5 volumes of extraction buffer containing 50 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 1 mM sodium ortho-vanadate, 10 mM sodium fluoride, 10% glycerol, 0.5% Triton-X, and a protease inhibitor cocktail (complete Mini, EDTA-free, Roche). The slurry was mixed on a rotating shaker for 10 minutes at 4^oC, followed by centrifugation (15,000 xg, 30 min, 4^oC). Protein in the supernatant was quantified using the BCA (bicinchoninic acid) assay (Pierce).

ICAT Labeling

BTH-treated and untreated proteome samples were extracted from four-week old Arabidopsis Col-0, *trx3*, *trx5* and *trx3/5* and diluted to a 2 mg protein/ml solution in 50 mM HEPES pH 7.5. Each sample was denatured in Denaturing Alkylation Buffer (DAB) containing 6 M urea, 0.5% SDS, 10 mM EDTA, 200 mM Tris-HCl, pH 8.5. Reduced cysteines were labeled with light ICAT (^{12}C) reagent under anaerobic and dark environment. After reduction of oxidized thiols with 50 mM TCEP, oxidatively modified cysteines were labeled with heavy ICAT (^{13}C) reagent. The labeled samples were subjected to overnight trypsin digestion. The biotinylated peptides were purified by cation exchange and avidin affinity column. Dried peptide pellets were then dissolved in cleavage buffer (Applied Biosystems) to remove the biotin from the ICAT tag. Cleaved samples were then subjected to LC-MS analysis**.**

References

Abramovitch, R. B., Anderson, J. C., & Martin, G. B. (2006). Bacterial elicitation and evasion of plant innate immunity. *Nature reviews. Molecular cell biology*, *7*(8), 601–11. doi:10.1038/nrm1984

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., … Ecker, J. R. (2003). Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science (New York, N.Y.)*, *301*(5633), 653–7. doi:10.1126/science.1086391

Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W.-L., Gomez-Gomez, L., … Sheen, J. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature*, *415*(6875), 977–83. doi:10.1038/415977a

Audenaert, K., Meyer, G. B. De, & Ho, M. M. (2002). Abscisic Acid Determines Basal Susceptibility of Tomato to Botrytis cinerea and Suppresses Salicylic Acid-Dependent Signaling Mechanisms 1, *128*(February), 491–501. doi:10.1104/pp.010605.1

Berrocal-Lobo, M., Molina, A., & Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. *The Plant journal : for cell and molecular biology*, *29*(1), 23–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12060224

Boller, T., & Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual review of plant biology*, *60*, 379–406. doi:10.1146/annurev.arplant.57.032905.105346

Buchanan, B. B., & Balmer, Y. (2005). Redox regulation: a broadening horizon. *Annual review of plant biology*, *56*, 187–220. doi:10.1146/annurev.arplant.56.032604.144246

Cordell, H. J. (2002). Epistasis : what it means , what it doesn ’ t mean , and statistical methods to detect it in humans, *11*(20), 2463–2468.

Cornelis, G. R. (2000). A SSEMBLY AND F UNCTION OF T YPE III S ECRETORY S YSTEMS.

Dangl, J. L., & Jones, J. D. G. (2001). defence responses to infection, *411*(June).

Dou, D., & Zhou, J.-M. (2012). Phytopathogen effectors subverting host immunity: different foes, similar battleground. *Cell host & microbe*, *12*(4), 484–95. doi:10.1016/j.chom.2012.09.003

Du, Y.-Y., Wang, P.-C., Chen, J., & Song, C.-P. (2008). Comprehensive functional analysis of the catalase gene family in Arabidopsis thaliana. *Journal of integrative plant biology*, *50*(10), 1318–26. doi:10.1111/j.1744-7909.2008.00741.x

Durrant, W. E., & Dong, X. (2004). Systemic acquired resistance. *Annual review of phytopathology*, *42*, 185–209. doi:10.1146/annurev.phyto.42.040803.140421

El Oirdi, M., El Rahman, T. A., Rigano, L., El Hadrami, A., Rodriguez, M. C., Daayf, F., … Bouarab, K. (2011). Botrytis cinerea manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *The Plant cell*, *23*(6), 2405–21. doi:10.1105/tpc.111.083394

FAO. (2013). *The state of food and agriculture, 2013. Lancet* (Vol. 2, pp. 313–4). Retrieved from http://www.fao.org/docrep/018/i3300e/i3300e00.htm

Ferrari, S., Plotnikova, J. M., De Lorenzo, G., & Ausubel, F. M. (2003). Arabidopsis local resistance to Botrytis cinerea involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *The Plant Journal*, *35*(2), 193–205. doi:10.1046/j.1365-313X.2003.01794.x

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M. G., … Box, P. O. (1996). A benzothiadiazole derivative induces systemic acquired resistance in tobacco, *10*(April), 61–70.

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., … Dong, X. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature*, *486*(7402), 228–32. doi:10.1038/nature11162

Gelhaye, E., Rouhier, N., Navrot, N., & Jacquot, J. P. (2005). The plant thioredoxin system. *Cellular and molecular life sciences : CMLS*, *62*(1), 24–35. doi:10.1007/s00018-004-4296-4

Gilbert, B. M., & Wolpert, T. J. (2013). Characterization of the LOV1-Mediated, Victorin-Induced, Cell-Death Response with Virus-Induced Gene Silencing. *Molecular plant-microbe interactions : MPMI*, *26*(8), 903–17. doi:10.1094/MPMI-01-13-0014-R

Gimenez-Ibanez, S., & Solano, R. (2013). Nuclear jasmonate and salicylate signaling and crosstalk in defense against pathogens. *Frontiers in plant science*, *4*(April), 72. doi:10.3389/fpls.2013.00072

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual review of phytopathology*, *43*, 205–27. doi:10.1146/annurev.phyto.43.040204.135923

Glazebrook, J., Zook, M., Mert, I. F., Kagan, I., Rogers, E. E., Crute, I. R., … Ausubelt, F. M. (1997). Phytoalexin-Deficient Mutants of Arabidopsis Reveal That PAD4 Encodes a Regulatory Factor and That Four PAD Genes Contribute to Downy Mildew Resistance. *Genetics*, *146*, 381–392.

Görlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K. H., … Ryals, J. (1996). Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *The Plant cell*, *8*(4), 629–43. doi:10.1105/tpc.8.4.629

Grant, S. R., Fisher, E. J., Chang, J. H., Mole, B. M., & Dangl, J. L. (2006). Subterfuge and manipulation: type III effector proteins of phytopathogenic bacteria. *Annual review of microbiology*, *60*, 425–49. doi:10.1146/annurev.micro.60.080805.142251

Ham, H., Sreelatha, A., & Orth, K. (2011). Manipulation of host membranes by bacterial effectors. *Nature reviews. Microbiology*, *9*(9), 635–46. doi:10.1038/nrmicro2602

Hisabori, T., Hara, S., Fujii, T., Yamazaki, D., Hosoya-Matsuda, N., & Motohashi, K. (2005). Thioredoxin affinity chromatography: a useful method for further understanding the thioredoxin network. *Journal of experimental botany*, *56*(416), 1463–8. doi:10.1093/jxb/eri170

Huang, H.-E., Ger, M.-J., Yip, M.-K., Chen, C.-Y., Pandey, A.-K., & Feng, T.-Y. (2004). A hypersensitive response was induced by virulent bacteria in transgenic tobacco plants overexpressing a plant ferredoxin-like protein (PFLP). *Physiological and Molecular Plant Pathology*, *64*(2), 103–110. doi:10.1016/j.pmpp.2004.05.005

Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. *Nature*, *444*(7117), 323–9. doi:10.1038/nature05286

Katti, S. K., Lemasterf, D. M., & Eklund, H. (1990). Crystal Structure of Thioredoxin from Escherichia codi at l-68 A Resolution. *J. Mol. Biol.*, *212*, 167–184.

Laloi, C, Rayapuram, N., Chartier, Y., Grienenberger, J. M., Bonnard, G., & Meyer, Y. (2001). Identification and characterization of a mitochondrial thioredoxin system in plants. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(24), 14144–9. doi:10.1073/pnas.241340898

Laloi, Christophe, Mestres-ortega, D., Marco, Y., Meyer, Y., & Reichheld, J. (2004). The Arabidopsis Cytosolic Thioredoxin h5 Gene Induction by Oxidative Stress and Its

W-Box-Mediated Response to Pathogen Elicitor 1, *134*(March), 1006–1016. doi:10.1104/pp.103.035782.al.

Lawton, K. a, Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., … Ryals, J. (1996). Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. *The Plant journal : for cell and molecular biology*, *10*(1), 71–82. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8758979

Leichert, L. I., Gehrke, F., Gudiseva, H. V, Blackwell, T., Ilbert, M., Walker, A. K., … Jakob, U. (2008). Quantifying changes in the thiol redox proteome upon oxidative stress in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(24), 8197–202. doi:10.1073/pnas.0707723105

Lin, Y.-H., Huang, H.-E., Wu, F.-S., Ger, M.-J., Liao, P.-L., Chen, Y.-R., … Feng, T.-Y. (2010). Plant ferredoxin-like protein (PFLP) outside chloroplast in Arabidopsis enhances disease resistance against bacterial pathogens. *Plant science : an international journal of experimental plant biology*, *179*(5), 450–8. doi:10.1016/j.plantsci.2010.07.006

Lindemann, C., & Leichert, L. I. (2012). Quantitative Redox Proteomics: The NOxICAT Method. (K. Marcus, Ed.)*Quantitative Methods in Proteomics*, *893*, 387–403. doi:10.1007/978-1-61779-885-6

Loon, L. Van, & Strien, E. Van. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *... and Molecular Plant ...*, 85–97. Retrieved from http://igitur-archive.library.uu.nl/bio/2002-0103-100912/UUindex.html

Lorang, J. (2012). Tricking the Guard : Exploiting Plant, *659*. doi:10.1126/science.1226743

Lorang, J., Kidarsa, T., Bradford, C. S., Gilbert, B., Curtis, M., Tzeng, S.-C., … Wolpert, T. J. (2012a). Tricking the guard: exploiting plant defense for disease susceptibility. *Science (New York, N.Y.)*, *338*(6107), 659–62. doi:10.1126/science.1226743

Lorang, J., Kidarsa, T., Bradford, C. S., Gilbert, B., Curtis, M., Tzeng, S.-C., … Wolpert, T. J. (2012b). Tricking the guard: exploiting plant defense for disease susceptibility. *Science (New York, N.Y.)*, *338*(6107), 659–62. doi:10.1126/science.1226743

Lorang, J. M., Sweat, T. a, & Wolpert, T. J. (2007). Plant disease susceptibility conferred by a “resistance” gene. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(37), 14861–6. doi:10.1073/pnas.0702572104

Malamy, J., Carr, J. P., Klessig, D. F., & Raskin, I. (1990). Salicylic Acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science (New York, N.Y.)*, *250*(4983), 1002–4. doi:10.1126/science.250.4983.1002

Marchand, C., Le Maréchal, P., Meyer, Y., Miginiac-Maslow, M., Issakidis-Bourguet, E., & Decottignies, P. (2004). New targets of Arabidopsis thioredoxins revealed by proteomic analysis. *Proteomics*, *4*(9), 2696–706. doi:10.1002/pmic.200400805

Mou, Z., Fan, W., & Dong, X. (2003, June 27). *Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell*. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12837250

Mpmi, G., Gilbert, B. M., & Wolpert, T. J. (2013). Characterization of the LOV1-mediated, Victorin-induced cell death response with Virus-Induced Gene Silencing. *Molecular Plant-Microbe Interactions*, 1–71.

Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., & Van Wees, S. C. M. (2012). Hormonal modulation of plant immunity. *Annual review of cell and developmental biology*, *28*, 489–521. doi:10.1146/annurev-cellbio-092910-154055

Rasmussen, J. B., Hammerschmidt, R., & Zook, M. N. (1991). Systemic Induction of Salicylic Acid Accumulation in Cucumber after Inoculation with Pseudomonas syringae pv syringae. *Plant physiology*, *97*(4), 1342–7. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1081169&tool=pmcentrez&rendertype=abstract

Reichheld, J.-P., Mestres-Ortega, D., Laloi, C., & Meyer, Y. (2002). The multigenic family of thioredoxin h in Arabidopsis thaliana: specific expression and stress response. *Plant Physiology and Biochemistry*, *40*(6-8), 685–690. doi:10.1016/S0981-9428(02)01406-7

Schenk, P. M., Kazan, K., Wilson, I., Anderson, J. P., Richmond, T., Somerville, S. C., & Manners, J. M. (2000). Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(21), 11655–60. doi:10.1073/pnas.97.21.11655

Stotz, H. U., Pittendrigh, B. R., Kroymann, J., Weniger, K., Fritsche, J., Bauke, a, & Mitchell-Olds, T. (2000). Induced plant defense responses against chewing insects. Ethylene signaling reduces resistance of Arabidopsis against Egyptian cotton worm but not diamondback moth. *Plant physiology*, *124*(3), 1007–18. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=59200&tool=pmcentrez&rendertype=abstract

Strange, R. N., & Scott, P. R. (2005). Plant disease: a threat to global food security. *Annual review of phytopathology*, *43*(Figure 1), 83–116. doi:10.1146/annurev.phyto.43.113004.133839

Sweat, T. a, & Wolpert, T. J. (2007). Thioredoxin h5 is required for victorin sensitivity mediated by a CC-NBS-LRR gene in Arabidopsis. *The Plant cell*, *19*(2), 673–87. doi:10.1105/tpc.106.047563

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., … Dong, X. (2008). Plant immunity requires conformational changes [corrected] of NPR1 via S-nitrosylation and thioredoxins. *Science (New York, N.Y.)*, *321*(5891), 952–6. doi:10.1126/science.1156970

Xie, D. (1998). COI1: An Arabidopsis Gene Required for Jasmonate-Regulated Defense and Fertility. *Science*, *280*(5366), 1091–1094. doi:10.1126/science.280.5366.1091

Yasuomi Tada, Steven H. Spoel, Karolina Pajerowska-Mukhtar, Zhonglin Mou, Junqi Song, Chun Wang, Jianru Zuo, X. D. (2008). Plant Immunity Requires Conformational Charges of NPR1 via S-Nitrosylation and Thioredoxins Yasuomi Tada. *Science*, *321*(952). doi:10.1126/science.1156970

Zimmerli, L., Métraux, J. P., & Mauch-Mani, B. (2001). beta-Aminobutyric acid-induced protection of Arabidopsis against the necrotrophic fungus Botrytis cinerea. *Plant physiology*, *126*(2), 517–23. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=111145&tool=pmcentrez&rendertype=abstract