INTERACTOME OF MAP KINASE 4 AND THIOREDOXIN IN PLANTS

By

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To my parents, my wife and our son Mark
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<td>Abscisic acid</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AVR</td>
<td>Avirulence gene/protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CBB</td>
<td>Coomassie brilliant blue</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>Cfu</td>
<td>Colony forming units</td>
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<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
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<tr>
<td>dpi</td>
<td>days post inoculation</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>EDTA</td>
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<td>FLS2</td>
<td>Flagellin sensing 2</td>
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<td>HR</td>
<td>Hypersensitive response</td>
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<td>IAM</td>
<td>Iodoacetamide</td>
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<td>JA</td>
<td>Jasmonic acid</td>
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<tr>
<td>kD</td>
<td>KiloDalton</td>
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<tr>
<td>m/z</td>
<td>Mass to charge</td>
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<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>MAPKK, MEK, MKK</td>
<td>MAPK kinase</td>
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<td>MAPKKK, MEKK</td>
<td>MAPK kinase kinase</td>
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<td>MS</td>
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<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PMSF</td>
<td>Phenylmethanesulfonylfluoride</td>
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<td>Abbreviation</td>
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<tr>
<td>PR</td>
<td>Pathogenesis related</td>
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<td>PRR</td>
<td>Pattern recognition receptor</td>
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<td><em>Pst</em></td>
<td><em>Pseudomonas syringae pv. tomato</em></td>
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<td>PTI</td>
<td>PAMP-triggered immunity</td>
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<td>R gene/protein</td>
<td>Resistance gene/protein</td>
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<td>RIN4</td>
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<td>RLK</td>
<td>Receptor like kinase</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>Y2H</td>
<td>Yeast two hybrid</td>
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INTERACTOME OF MAP KINASE 4 AND THIOREDOXIN IN PLANTS

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Protein-protein interaction (PPI) plays a fundamental role in plants to orchestrate transcriptional, translational and metabolic activates. Genetic and biochemical tools have been used to study PPIs at the individual protein level. It is increasingly important to characterize PPIs at large-scale in the post-genomic era because identification of targets of key proteins in plant signal transduction is crucial for better understanding the molecular networks. Unbiased high-throughput proteomics was employed here to study the interactome of two proteins in plant redox regulation: mitogen-activated protein kinase 4 (MPK4) and thioredoxin h. MPK4 is not only involved in both the homeostasis and signaling of reactive oxygen species (ROS), but also interacts with a broad range of substrates whose precise identity remains poorly defined. To elucidate how MPK4 functions in plants, immunoprecipitation coupled with mass spectrometry was used to identify putative MPK4 interacting proteins. The candidate list includes stress responsive proteins, proteins involved in ROS homeostasis and transcription factors, providing further insight into the molecular roles of MPK4 in different cellular processes. MPK4 showed a dynamic phosphorylation pattern both within and outside of the conserved TEY activation loop during its activation by fllg22, indicating a potential role of
phosphorylation as a mechanism in regulating MPK4 activity. Moreover, perturbation of MPK4 activity resulted in aberrant ROS production, since both *Arabidopsis thaliana* stably expressing MPK4 and *Nicotiana benthamiana* transiently expressing a constitutive MPK4 showed higher level of ROS accumulation. Thioredoxins are small proteins that reduces redox sensitive proteins through disulfide exchange, thus counterbalance the oxidation modifications by ROS. Although hundreds of Trx targets have been identified using different approaches, the capture of targets in a quantitative and efficient manner is challenging. A high-throughput method using cysteine-reactive tandem mass tag (cysTMT) labeling followed by liquid chromatography -mass spectrometry to screen for Trx *h* targets was developed. Application of this method in guard cell-enriched epidermal peels from *Brassica napus* revealed 80 Trx *h* targets involved in a broad range of processes, including photosynthesis, stress response, metabolism and cell signaling. These results demonstrated the power of proteomics in the discovery of novel PPIs.
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

MAPK Cascades are Essential Signaling Components in Plants

Overview of MAPK Cascades in Plants

Mitogen-activated protein kinase (MAPK) cascades are highly conserved in eukaryotes (Andreasson and Ellis, 2010; Pitzschke, 2015). It has been shown that plant MAPKs play essential roles in responses to environmental stress (Cheong and Kim, 2010; Meng and Zhang, 2013; Sinha et al., 2011). Knowledge of mechanisms underlying stress tolerance could be used to improve the overall fitness and productivity of plants. Both environmental cues and developmental signals can trigger the activation of MAPK cascades (Meng and Zhang, 2013; Opdenakker et al., 2012; Sinha et al., 2011; Xu and Zhang, 2015). Typically, a MAPK kinase kinase (MAPKKK, or MEKK) is first activated, which in turn phosphorylates and activates a MAPK kinase (MAPKK, MKK, or MEK). The activated MAPKK switches on a MAPK, the terminal protein kinase in the cascade, which interacts and phosphorylates a diverse array of substrates in a spatially and temporally specific manner to transduce the upstream signals (Meng and Zhang, 2013; Opdenakker et al., 2012; Pitzschke, 2015; Xu and Zhang, 2015). Thus, highly ordered PPI underlie the MAPK cascade-mediated signaling events.

There are 60 MAPKKK, 20 MAPKK and 10 MAPK genes in the model plant Arabidopsis thaliana (Ichimura et al., 2002). Genomic analyses for MAPKs indicate similar numbers in other plant species (Hamel et al., 2006; Mohanta et al., 2015).

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Theoretically, various combinations of these enzymes could form numerous cascades to deal with developmental signals as well as with various biotic and abiotic stresses. However, genetic evidence does not support a random combination of MAPK components in a cascade (Xu and Zhang, 2015). Instead, specific sets of cascade components are thought to have developed during evolution, resulting in a limited number of different MAPK cascades (Pitzschke, 2015; Xu and Zhang, 2015). This concept is also supported by large-scale screening and validation of MAPK targets. For example, experiments using yeast-two-hybrid (Y2H) (Lee et al., 2008) and protein microarrays (Popescu et al., 2009) indicated multiple interactions between different M KK-M PK pairs and between various MPKs and potential substrates in Arabidopsis. Yet, further in vitro kinase assays using recombinant proteins showed no or only weak phosphorylation between many of the identified interaction pairs (Popescu et al., 2009). The physiological relevance of putative interactions between M KK-M PK remains to be determined. Only a handful of MAPK cascades have been experimentally verified. While cascades involving MPK3/6 in group A and MPK4 in group B of the MPK family, respectively, (Bethke et al., 2009; Gao et al., 2008; Suarez-Rodriguez et al., 2007; Wang et al., 2007) have been well-characterized, there are only a few studies on MAPKs from the C and D groups (Doczi et al., 2007; Ichimura et al., 2002).

The identified handful of experimentally determined MAPK cascades are outnumbered by the cellular processes they regulate. How can so few cascades relay signals in so many cellular processes? One explanation is the spatiotemporal nature of interactions that occur among the players. Unique expression of substrates in specific types of cells represents one mechanism to achieve signal specificity. For example,
SPEECHLESS (SPCH), a transcription factor phosphorylated by MPK6, is only expressed in stomatal lineage cells to initiate the differentiation of guard cell mother cells into guard cells (Lampard et al., 2008). Alternatively, specific expression of MAPKs also governs downstream signaling. For instance, the preferential expression of MPK9 and MPK12 in guard cells underlies their cell-specific function in ABA signaling (Jammes et al., 2009). In addition, different signals may direct cell-specific MAPKs to interact with distinct substrates, leading to different physiological events. Moreover, phosphorylation itself is a way to diversify protein functions. Addition of the bulky negatively-charged phosphate group typically results in significant changes in properties of the substrates, such as protein stability and enzymatic activity (Lampard et al., 2008; Liu and Zhang, 2004), subcellular localization (Roux et al., 2015), and ability to bind other molecules (Li et.al, 2015). The changes can lead to modulation of downstream signaling, such as gene expression (Li et al., 2015a), protein turnover (Liu and Zhang, 2004), ion channel activity (Zhang et al., 2014), and redox homeostasis (Kong et al., 2012), thus allowing for regulation of different cellular processes.

Knowledge gaps for the known plant MAPK cascades mainly result from a lack of information on the substrates. Traditionally, genetic interaction has been used to identify MAPK cascade components one protein at a time (Lampard et al., 2008; Liu and Zhang, 2004; Roux et al., 2015). Large-scale screening methods for MAPK substrates have also been developed (Andreasson et al., 2005; Feilner et al., 2005; Lee et al., 2008). In addition, various kinase assays have been employed to show a phosphorylation reaction within a kinase-substrate pair. The phosphorylation-dependent properties of the putative substrate may be revealed by amino acid mutagenesis at specific
phosphorylation sites. A number of methods have also been used to functionally characterize the substrates in vivo. Furthermore, target proteins of MAPK may be predicted, taking advantage of the fact that MAPKs are Pro-directed Ser/Thr kinase (Whisenant et al., 2010). Recently, mass spectrometry (MS) based large-scale phosphoproteomics has been applied to identify kinase substrates in plants (Hoehenwarter et al., 2013; Umezawa et al., 2013; Wang et al., 2013). Exciting advances are revealing many more aspects of MAPK cascades in regulating plant development and adaptation to a changing environment.

**MAPK Cascade-Mediated Signaling Events**

Interaction of a MAPK with its substrate is usually triggered by a developmental or stress signal (Pitzschke, 2015; Xu and Zhang, 2015). Subsequent protein phosphorylation can be detected in many ways, such as a gel mobility shift assay. For example, treatment of Arabidopsis plants for 3 min with flg22, a pathogen-associated molecular pattern (PAMP), resulted in retarded mobility in SDS-PAGE of the Arabidopsis SH4-related 3 protein (ASR3). To show that a band shift is caused by phosphorylation, treatment with lambda protein phosphatase abolishes the mobility shift by removing phosphate from phosphorylated Ser or Thr residues (Li et al., 2015a). Another gel-based mobility shift assay utilizes the Phos-tag reagent, which covalently modifies phosphorylated proteins and retards their mobility (Bethke et al., 2009; Mao et al., 2011; Zhang et al., 2001). Typically, an immunoblot with antibodies directed against the protein of interest is used to verify the identity of the shifted band. If such antibodies are not available, expression of epitope-tagged proteins in transgenic plants or protoplasts provides an alternative strategy (Li et al., 2015a). Antibodies directed
against either the protein or the epitope tag can be used to pull down the protein of interest, and the isolated protein can be used for further characterization.

Precise localization of the phosphorylation sites is usually the first step in understanding the mechanism of protein kinase mediated signaling. To this end, mapping of the phosphorylation sites of a putative substrate is often performed before detailed studies determining the physiological effects of the substrate phosphorylation. This is typically done using the immuno-precipitated (IP) protein for analysis by MS-based method. Thus, phosphorylation events can be pinpointed at the specific amino acid residue level. A good indication that the protein is a direct target of MAPK phosphorylation is if the phosphorylated residue is followed by a Pro. The identified phosphorylation site(s) can be further mutated to Ala or Asp/Glu residue(s) to create phospho-inactive or presumably constitutively active mutants (phosphomimic), respectively. A mobility shift assay can be performed with such a mutant to confirm that retarded mobility is caused by specific phosphorylated residues. A caveat for this assay is that not all phosphorylation events trigger discernible mobility shifts on a SDS-PAGE, probably due to the little to no change in size of the phosphoproteins. The \textit{in vivo} effects of phosphorylation on protein properties such as stability, activity, and subcellular localization may be addressed using various approaches depending on the substrate in question (Figure 1-1).

\textbf{MAPKs Function via Interaction with Specific Substrates}

\textbf{General Strategies for Identifying MAPK-Substrate Interaction}

To determine possible MAPK-substrate pairs, one can start with either a MAPK or a substrate (Figure 1-1B, left panel). On a large scale (Figure 1-1B, left panel), Y2H screens using MAPKs as bait (Andreasson et al., 2005) and protein microarrays with
1,690 Arabidopsis proteins on a chip (as potential substrates) (Feilner et al., 2005) have been employed to identify MAPK substrates. In addition, direct labeling of kinase substrates using an analog of adenosine 5'-triphosphate (ATP) and analog-sensitive kinase have been reported (Chi and Clurman, 2010; Kumar et al., 2004). In this approach, the kinase of interest is mutated in the ATP binding pocket so that it binds to ATP analogs such as ATP-γ-S. The mutated kinase is able to utilize ATP-γ-S to thiophosphorylate direct substrates, which can be trypsin-digested. The resulting thiophosphorylated peptides can be purified by sulfoLink beads and then identified by MS-based methods. This approach has been applied to MAPK substrate identification in yeast (Westfall and Thorner, 2006) and mammalian systems (Carlson et al., 2011; Smith et al., 2014), but not yet in plants. In another approach, screening of a synthetic peptide library by in vitro kinase assays, followed by Edman sequencing (Yaffe, 2004) or MS-based peptide identification (Trinh et al., 2013) identifies putative kinase substrates. Box 1 (listed as Appendix) summarizes the available approaches for screening of MAPK substrates. Given the diverse nature of MAPK substrates in different cellular processes, it is unlikely that one screening method will capture all the potential targets. Thus, complimentary methods would provide multiple lines of evidence of true MAPK-substrate interactions.

On the other hand, if a certain phosphorylation event can be linked to MAPK activity, the responsible kinase can be found relatively easily (Figure 1-1B, right panel). In this case, the application of MAPK cascade inhibitors such as U0126 is usually used to show the involvement of a MAPK in a phosphorylation event (Li et al., 2015a). The requirement of MAPKs in a phosphorylation event could be strengthened by
coexpression of MAPK phosphatase, which would abolish the band shift of the substrate. Since there are only 20 MPks in Arabidopsis, an individual MPK can be expressed in a transient system such as protoplasts that respond to the stimulus of interest. Treatment of the cells with the stimulus followed by an in vitro kinase assay with the known substrate could be used to identify the upstream MPK as the activated kinases can be extracted in the activated state because they are covalently modified (Li et al., 2015a).

Commonly used methods in PPI are powerful tools to test putative MAPK-substrate interactions (Figure 1-1B, middle panel). For example, co-immunoprecipitation (Co-IP), co-localization using green fluorescent protein (GFP) and yellow fluorescent protein (YFP), bimolecular fluorescence complementation (BiFC), and fluorescence imaging such as fluorescence resonance energy transfer (FRET) have been employed (Table 1). The interaction of a putative MAPK-substrate can be validated further by in vitro and in vivo kinase assay. Recombinant proteins are commonly used to test the phosphorylation reaction. Typically, His- or GST-tagged MAPK and substrates are expressed in and then purified from E. coli, followed by an in vitro kinase assay to show the incorporation of $\gamma^{32}$P into the substrates. To validate the interactions in planta, coexpression of tagged MAPK and its substrate in protoplasts (Li et al., 2015a), infiltrated leaves (Oh et al., 2013) or stable transgenic plants (Roux et al., 2015) have been reported. More stringently, a tagged substrate protein can be expressed in a stable transgenic plant under its own promoter without overexpressing the kinase of interest (Kong et al., 2012; Roux et al., 2015). This helps prevent artifacts resulting from
high expression levels of the kinase. Co-immunoprecipitation (Co-IP) is typically used to confirm that the two proteins interact *in planta*.

Both *in vitro* and *in vivo* protein kinase activity assays are valuable, although differences in results are commonly seen. One reason is that the expression level of protein kinases in plants is usually low as compared to the high amount of proteins used *in vitro*. Thus, a kinase may lose its specificity due to the high ratio of kinase to substrate *in vitro* and promote phosphorylation of poor substrates. In addition, *in vitro* assays may bring together proteins that never interact *in vivo*, leading to promiscuous phosphorylation. Another likely explanation for the differences is the absence of scaffold proteins in *in vitro* kinase assays. Both MAPK signal amplitude and duration as well as cell-type specific activation could be under the control of scaffold proteins. Although scaffold proteins that bind MAPK cascade components have been identified in yeast (Good et al., 2009) and animal systems (Dhanasekaran et al., 2007), scaffold proteins in plants are generally poorly understood. One recently described scaffold protein from Arabidopsis and named for its mammalian counterpart is RACK1 (receptor for activated C kinase 1). In plants this protein binds to all members in the MEKK1-MKK4/5-MPK3/6 cascade and β subunit of G-protein (Cheng et al., 2015).

The highly conserved feature of MAPK cascades enables use of heterologous species and reagents for identifying plant MAPKs. Functional complementation of the salt-sensitive yeast strain *pbs2Δhog1Δ*, where both MAPKK (PBS2, ortholog of MKK2 in Arabidopsis) and MAPK (HOG1, ortholog of MPK6 in Arabidopsis) were mutated, resulted in a successful identification of a constitutively active Arabidopsis MPK6 (Berriri et al., 2012). In addition, anti-phospho-ERK1 antibody, commercially known as
phospho-p44/42 that was generated against phosphorylated human p44 MAPK (ERK1), is widely used in plant research to detect phosphorylated MPK6, MPK3 and MPK4 simultaneously (Berriri et al., 2012; Cheng et al., 2015; Kong et al., 2012; Xu et al., 2014; Zhang et al., 2015).

MAPK Substrates in Multiple Plant Signaling Events

A number of stresses activate MPK3/6 and MPK4 in Arabidopsis (Gao et al., 2008; Gudesblat et al., 2007; Petersen et al., 2000; Xu et al., 2014). These three MAPKs and their orthologs have been most extensively studied. MPK3 and MPK6 overlap in numerous processes, with MPK6 playing a more important role in aspects such as inflorescence architecture and defense against fungal pathogen (Andreasson and Ellis, 2010; Galletti et al., 2011; Meng et al., 2012; Pitzschke, 2015). Both are activated by MKK4/5, and they share many common substrates. The redundancy is also reflected by the lethal phenotype exhibited in mpk3/mpk6 double mutant, but not exhibited in either single mutant (Wang et al., 2007). MPK4 was initially considered to be a negative regulator in plant defense, since the Arabidopsis mpk4 mutant shows constitutively active defense responses such as higher pathogen-related gene expression and increased resistance to pathogens (Peterson et al., 2000). More roles of MPK4 were realized as a number of MPK4 targets functioning in plant defense have been experimentally characterized, (Andreasson et al., 2005), cytokinesis (Sasabe et al., 2011), mRNA stability (Roux et al., 2015) and regulation of DNA binding activity (Li et al., 2015a). During the last several decades, many kinase-substrate interaction pairs have been established (Table 1). It has been also realized that a single protein kinase could function in multiple biological processes by interacting with various substrates, including transcription factors, enzymes and repressors (Figure 1-2 and Table 1-1).
However, in most cases, it remains to be determined how each upstream signal activates the corresponding substrate and to what extent activation of MAPKs affects the phosphorylation status of their substrates.

**MPK6 Substrates**

The first MAPK-substrate pair identified in plants was MPK6-ACS6 (1-aminocyclopropane-1-carboxylic acid synthase) in ethylene biosynthesis (Liu and Zhang, 2004). As increases of ACS activity and ethylene synthesis were observed at the same time following MPK6 activation, Liu and Zhang connected the two events by showing direct phosphorylation of ACS6 by MPK6 in vivo. They showed that both recombinant and native MPK6 can phosphorylate ACS6, while no phosphorylation of ACS6 can be detected in the mpk6 mutant. In addition, a decrease in mobility of ACS6 was detected after MPK6 activation in transgenic plants. The authors also showed that phosphorylation on ACS6 by MPK6 increased its stability and thus the activity of ACS6.

Tobacco SIPK/WIPK, orthologs of MPK3/6 in Arabidopsis, are activated during the defense response to fungal infection that leads to camalexin production (Ren et al., 2008). WRKY transcription factors were shown to be potential targets of these MAPKs by monitoring camalexin accumulation in Arabidopsis wrky mutants expressing a constitutively active mutant of MEK2 (MEK$^{DD}$), the MAPKK that phosphorylates and activates SIPK/WIPK. MPK3/6-dependent camalexin accumulation was impaired in wrky33, but not in other wrky mutants (Mao et al., 2011). To gain insight into whether MPK3/6 directly interacted with WRKY33, an in vitro kinase assay was performed with recombinant proteins. Strong phosphorylation of WRKY33 was observed by both activated MPK3 and MPK6. In addition, evidence of MPK3/6-WRKY33 as a kinase-substrate pair came from the fact that WRKY33 showed retardation in a Phos-tag...
mobility assay in MEK\textsuperscript{DD} plants. However, WRKY\textsuperscript{33SA}, a mutant in which five Ser were mutated to Ala, did not show the mobility shift under the same condition (Mao et al., 2011). Interestingly, WRKY33 could also be regulated by other kinases in pathogen-induced camalexin production. For instance, WRKY33 was released from the MPK4/MKS1 complex (see MPK4 section for detail) to activate expression of camalexin synthetic genes in response to bacterial infection (Qiu et al., 2008a).

Spatiotemporal-specific interaction between kinase and substrates provides a mechanism by which one MAPK can perform multiple roles. The first MAPK substrate identified in plant development was SPCH, a transcription factor that controls guard cell differentiation (Lampard et al., 2008). The idea that SPCH could be a MPK3/6 substrate was deduced from the similarity of the phenotype of the \textit{spch} loss-of-function mutant to that of plants with constitutive MPK3/6 activation. An \textit{in vitro} kinase assay showed that SPCH was phosphorylated by MPK3/6, and the domain interacting with the MAPKs was identified. In addition, mutation of the Ser and Thr residues to Ala residues within the MAPK-interacting domain promotes SPCH activity and thus additional cell division. This study provides an example where MAPKs negatively regulate transcription factor stability and activity, enabling fine-tuning of plant development in response to environmental cues. Thus, regulation of target stability is one of the means by which MAPKs control cellular activity. Yet, the modification could have opposite effects: stabilizing in the case of ACS6 and destabilizing for SPCH (Lampard et al., 2008; Liu and Zhang, 2004).

MPK3 and MPK6 are functionally redundant in many physiological processes. However, distinct non-redundant roles of MPK6 have been described. For instance,
ethylene response factor 104 (ERF104) was shown to be phosphorylated in vivo by MPK6, but not by MPK3 (Bethke et al., 2009). The physical interaction between MPK6 and ERF104 in vivo was further confirmed by FRET. In addition, ERF104 with reduced mobility was detected when co-expressed with MKK5, which activates MPK6. Significantly, flg22-activated MPK6 was found to disrupt the MPK6-ERF104 complex by phosphorylation. Thus, this represents a mechanism where release of substrate from a preformed complex by MAPK phosphorylation is used to regulate downstream gene expression.

**MPK4 Substrates**

MAP kinase 4 substrate 1 (MKS1) was the first substrate of MPK4 identified and the second MAPK substrate discovered in plants. Full-length MPK4 was used as a bait to identify MPK4 interacting proteins using an Y2H analysis. MKS1 was found to interact with MPK4, but not with other MAPKs. Recombinant MKS1 from *E. coli* was phosphorylated by HA-tagged MPK4 expressed in Arabidopsis. Co-IP results showed that MPK4 interacts with MKS1 in vivo. Such an interaction was further confirmed by co-localization, and poor phosphorylation of MKS1 in the *mpk4* mutant (Andreasson et al., 2005). The authors also showed that MKS1 interacts with WRKY transcription factors. Therefore, MPK4 may use MKS1 as a mediator to control transcription factors and thus plant immunity responses. This idea was supported by two subsequent studies demonstrating that phosphorylation of MKS1 by MPK4 is a prerequisite for the release of WRKY33 and the activation of *Phytoalexin Deficient 3 (PAD3)* expression (Qiu et al., 2008a; Qiu et al., 2008b).

Although MPK4 was initially identified as a negative regulator in plant immunity (Petersen et al., 2000), the MEKK1-MKK1/MKK2-MPK4 cascade was also activated by
PAMPs (Gao et al., 2008; Suarez-Rodriguez et al., 2007), highlighting the dual role of
MPK4 in immunity. In addition, P. syringae effectors such as AvrB could interact with
MPK4 and induce the phosphorylation of MPK4 (Cui et al., 2012). Phosphorylation of
MPK4, but not other MPKs, was greatly enhanced in transgenic plants expressing AvrB.
Significantly, inoculation of plants with bacteria delivering AvrB also caused MPK4
phosphorylation. Co-IP assays demonstrated the interaction of the proteins in vivo in
transgenic plants expressing FLAG-tagged AvrB. Interestingly, interaction with and
activation of MPK4 mediated by AvrB was promoted by HSP90 chaperone protein (Cui
et al., 2012). Other pathogenic effectors such as HopAI1, however, inhibit MPK4
activity, leading to defense responses mediated by SUMM2 (suppressor of mkk1 mkk2
2, a nucleotide binding–leucine-rich repeat resistant protein) (Zhang et al., 2012). Thus,
the constitutive defense phenotype in mpk4 could be partially due to the activation of
SUMM2. However, the mechanisms by which SUMM2 is activated and mediates
defense responses remain to be determined.

In addition to MKS1, the Y2H screening showed another putative MPK4
substrate called protein associated with topoisomerase II 1 (PAT1), which is required for
the decapping of mRNAs in plants (Roux et al., 2015). The interaction was validated by
co-IP using transgenic Arabidopsis expressing both MPK4 and PAT1 with different
epitope tags. The in vivo phosphorylation site was mapped by MS/MS using isolated
PAT1 from flg22-treated plants. The phosphorylation activity of MPK4 and other MPKs
on PAT1 were also compared to show that PAT1 specifically interacts with MPK4.
Phosphorylation of PAT1 resulted in relocalization of the protein into cytoplasmic
processing bodies (foci involved in mRNA turnover in eukaryotes) to regulate mRNA
decay. Interestingly, MPK6 has also plays a role in mRNA turnover by interacting with mRNA decapping 1 (DCP1). Phosphorylation of DCP1 by MPK6 under dehydration stress promotes mRNA decapping (Xu et al., 2012).

A screen for suppressors of the constitutive defense phenotype of the mkk1/mkk2 double mutant in Arabidopsis gave rise to the identification of an unexpected MPK4 substrate (Kong et al., 2012). Suppressor of mkk1mkk2 1 (SUMM1) encodes MAPKKK9, which is also known as MEKK2. Genetic evidence showed that dwarfism in mpk4 is completely rescued by crossing mpk4 with summ1, where no functional SUMM1 is expressed. The genetic interaction between MPK4 and SUMM1 also led to an investigation of the interaction at the physical level using Y2H. The results showed that the N-terminal of SUMM1 interacts with and is phosphorylated by MPK4. Co-IP confirmed that they interact in vivo. Thus, MEKK2 acts as a direct target of MPK4, in contrast to the canonical MAPK cascades where MAPKKKs function upstream of MAPKs. A recently identified MPK4 substrate is ASR3, a transcription repressor closely related to shattering 4 (SH4), which is a quantitative trait locus controlling rice grain shattering (Li et al., 2015a). Similarly to the mpk4 mutant, asr3 showed enhanced pathogen resistance and immune gene expression. Treatment with U0126, a MAPK pathway inhibitor, significantly reduced flg22-induced ASR3 phosphorylation. Co-expression of ASR3 with various HA-tagged MAPKs in protoplasts showed that MPK4 strongly phosphorylates ASR3. Interestingly, flg22-triggered phosphorylation of ASR3 by MPK4 did not change its transcriptional repressor activity. Instead, its promoter-binding activity was enhanced by phosphorylation. Elevated promoter binding was also observed with the phospho-mimic mutant ASRT189D. Therefore, MPK4 may regulate
flg22-induced immune-related gene expression via phosphorylation of a transcription repressor.

There is a growing body of evidence to show that MPK4 is a multi-functional kinase, and it may play distinct roles through interactions with specific substrates. Another example supporting the multifunctional character of MPK4 is the finding that it plays an essential role in cytokinesis (Kosetsu et al., 2010). Not surprisingly, new substrates MAP65-1/2/3 in the phragmoplast were found and the interactions were confirmed by in vitro kinase assays (Beck et al., 2010; Sasabe et al., 2011).

Another intriguing finding from recent work is that MPK4 from different species may function differently (Berriri et al., 2012). For instance, silencing of MPK4 in Arabidopsis results in dwarfism, but silencing of MPK4 in Nicotiana attenuata causes a mild slow-growth phenotype (Hettenhausen et al., 2012). Notably, silencing MPK4 in N. attenuata resulted in highly impaired abscisic acid (ABA)-induced stomatal closure, while ABA signaling for stomatal closure is not affected in MPK4-silenced Nicotiana tabacum plants (Gomi et al., 2005). Thus, species-specific function of MAPKs may account for how MAPKs control multiple physiological processes.

**Interaction Domains of MAPKs and their Substrates**

As discussed above, functional studies of individual MAPK-substrate interactions reveal that substrate phosphorylation may result in different outcomes in terms of protein stability as well as activity. These observations raise the possibility that MAPKs contribute to many aspects of plant signaling via unique interaction with different substrates. Characterization of the nature of the interactions would reveal how MAPKs work biochemically. MAPKs have a common docking (CD) domain with a conserved Asp residue at the C-terminus. Interaction between the CD domain and docking sites
(DS) on MAPK interacting proteins determines the specificity of kinase-substrate interaction and efficiency of substrate phosphorylation (Tanoue and Nishida, 2002). Mutation of either the CD domain of MAPKs or the docking sites of substrates results in a failed MAPK-substrate interaction. The CD domains are conserved within the four plant MAPK groups, but they show variations among the groups (Mohanta et al., 2015). Interestingly, the CD domain is outside of the kinase catalytic domain (Tanoue and Nishida, 2002). Similarly, the DS of substrates is distinct from the phosphorylation site (Pitzschke, 2015). This suggests that the docking of a MAPK with its substrate occur at a site separate from the kinase active site and substrate phosphorylation site. Alternatively, mediators such as scaffold proteins may be involved in the docking and phosphorylation.

Comparison of the protein sequences of mammalian MAPK substrates shows certain conserved motifs within the DS (Pitzschke, 2015). Such a convergence is yet to be observed in plant MAPK substrates because their interacting domains have not been defined (Andreasson and Ellis, 2010). However, it is tempting to speculate that the substrates may have conserved binding domains in spite of the fact that they are proteins from diverse families, functioning in a plethora of distinct physiological events. In a few studies, domains of unknown function in substrates were tested for interaction with the MAPK. For example, both the N-terminal and the C-terminal domains of SUMM1 were analyzed for binding MPK4 by using Y2H (Kong et al., 2012), and they found that only the N-terminus, but not the conserved C-terminus of SUMM1, interacts with MPK4. In another study, truncated versions of MKS1 were analyzed in Y2H and activity assays, and it was found that MKS1 without the VQ motif cannot interact with
either MPK4 or WRKY (Andreasson et al., 2005). In another approach a single amino acid that is required for protein activity can also be mutated to verify the importance of the site. In AvrB, substitution of D297 by an Ala showed that D297 is essential for phosphorylation of this protein by MPK4, but not the interaction between them (Cui et al., 2010).

**Engineering Plants with MAPK/Substrate to Enhance Fitness**

As we learn more and more about MAPKs and their substrates, scientists have started to alter the expression levels and/or activities of these proteins toward enhancing plant fitness. For example, the finding that MPK4 is an important negative regulator in plant defense makes it an intriguing target for improving disease resistance. MPK4 in soybean (*Glycine max*) was silenced, and the resulting plants showed higher resistance to both downy mildew and mosaic virus. However, the silenced soybean also showed a decreased expression of genes in plant growth and development, leading to dwarf stature (Liu et al., 2011). Similarly, silencing MPK4 in *Nicotiana attenuata* also resulted in smaller plants with enhanced resistance to the bacterial pathogen *Pseudomonas syringae* pv. tomato (*Pst*) DC3000. Importantly, the silenced plants also showed increased photosynthesis rates and delayed senescence, and thus significantly increased seed production (Hettenhausen et al., 2012). In contrast, a study overexpressing *Brassica napus* MPK4 in *B. napus* also showed elevated resistance to a fungal pathogen *Sclerotinia sclerotiorum* (Wang et al., 2009). A possible explanation is that MPK4 is not only a negative regulator in plant systemic immune response, but also a positive player in jasmonic acid-mediated defense response. An alternative explanation could be the difference of the roles of MPK4 in different plant species. Thus,
it seems that the results of engineering plants with modified MPK4 levels are case-specific.

MPK4 substrates have also been altered in plants through genetics, resulting in different phenotypes. CaMV 35S promoter-driven expression of MKS1 led to semi-dwarf plants with higher resistance to the bacterial pathogen *Pst* DC3000 (Andreasson et al., 2005), but lower resistance to the fungi *Botrytis cinerea* (Fiil and Petersen, 2011). Thus, altering the expression level of MKS1 may render plants with specific resistance.

Another substrate of MPK4, ASR3, plays a negative role in disease resistance. Plants overexpressing both wild type and phosphomimic ASR3 proteins (ASR3<sup>T189D</sup>) were more susceptible to infection by bacterial pathogens, with the ASR3<sup>T189D</sup> mutant showing over 10-fold higher bacterial growth (Li et al., 2015a). Thus, it will be interesting to see whether the resistance to disease could be enhanced by knocking out ASR3 or rendering it unable to be phosphorylated.

The well-established role of AtMPK6 in plant defense also triggered the investigation of its ortholog in soybean. Overexpression of *GmMPK6* in Arabidopsis caused hypersensitive response (HR)-mimic cell death, while silencing of *GmMPK6* in soybean gave rise to enhanced resistance to downy mildew (Liu et al., 2014). In addition, MPK6 regulates drought stress through ACS-catalyzed ethylene production. In pioneering work, Meng et al. showed that the MPK6-ACS pathway is required in rose flower rehydration. *Rosa hybrida* MPK6-silenced plants showed reduced ethylene production during rehydration (Meng et al., 2014). MPK6 could also be overexpressed and activated for improvement in other important physiological processes such as phosphate (Pi) uptake. Lei et al. showed that low phosphate triggers MPK6 activation.
and that transgenic Arabidopsis with activated MPK6 shows enhanced Pi uptake and accumulation (Lei et al., 2014).

MAPKs regulate leaf senescence, an important and complex development phase in plants. Leaves of MPK6 knockout mutants have prolonged lifespan. In contrast, overexpressing a constitutively active MKK9 (an upstream kinase of MPK6) resulted in premature senescence both in leaves and in the whole plant (Zhou et al., 2009). Since MPK6 also regulates the ethylene pathway, and ethylene induces plant senescence (Liu and Zhang, 2004), it will be interesting to see whether ethylene acts as a mediator in MPK6 promoted plant senescence. Currently, the substrate of MPK6 in this process is unknown.

Redox Regulation by Thioredoxins

Thioredoxins Interact with Target Proteins via Thiol Disulfide Exchange

Under unfavorable conditions, MAPKs are activated to regulate plant defense responses. Many secondary messengers such as Ca\(^{2+}\) and ROS relay the signals as communication tools between the upstream environmental and developmental cues and the downstream cellular responses such as reprogramming of the transcriptome and regulation of protein turnover and activity (Kocsy et al., 2014). Of particular importance is the homeostasis of ROS. At low concentration, ROS serve as signaling components. However, they are toxic to plants at elevated concentration due to their high activity toward other molecules (Bykova et al., 2011; Jeong et al., 2011; Sheehan et al., 2010). For example, the thiol of Cys residues could be modified by ROS in a number of ways, due to the multiple oxidation states that sulfur can assume (from -2 in R-SH to +6 in SO\(_4^{2-}\)) (Montrichard et al., 2009). Importantly, modification of the Cys residues results in
significant changes in protein conformation, which is often accompanied by alteration in protein function (Meyer et al., 2008; Montrichard et al., 2009).

On the other hand, ROS burst-triggered modifications in redox states of many proteins could be reversed by molecules with reducing power. Thioredoxins (Trx) are a group of ubiquitous small proteins that transfer reducing power through its disulfide reductase activity within the conserved WCGPC motif (Meyer et al., 2008; Montrichard et al., 2009). After reducing target proteins, oxidized Trx can be regenerated by NADPH-dependent thioredoxin reductases or by ferredoxin-Trx reductase, thus providing a reaction cycle to continually maintain the cellular redox state. Thus, switch between a sulfhydryl group and disulfide bond on key cysteine residues of redox responsive proteins provide an effective mechanism for plant to modulate protein stability, activity, and binding pattern to adapt to the changing environment. Along with glutathione and glutaredoxins, Trxs are the major components to control the thiol redox status (Balmer et al., 2006a; Schurmann and Buchanan, 2008; Yoshida et al., 2015).

Regulation of substrate function via reduction of the disulfide bonds on target proteins by Trx has been well documented (Montrichard et al., 2009). The classic Trx target proteins are key enzymes in the Calvin cycle such as fructose-1,6-bisphosphatase (FBPase) and NADP-malate dehydrogenase (NADP-MDH), which are activated by light through Trx. Other enzymes within the chloroplast have also been determined as Trx regulated proteins, participating in photosynthesis, starch synthesis, and cellular redox state balancing (Montrichard et al., 2009; Yoshida et al., 2015). Therefore, Trx acts as the “biochemical eyes” to turn on and off the Calvin cycle in response to the diurnal and nocturnal cycle. Due to the complexity of the redox network
in chloroplast, five different types of Trx have been described so far in this organelle: $f$, $m$, $x$, $y$ and $z$. In addition, Trx $o$ has been discovered in mitochondria. The $h$ type of Trx ($h$ stands for heterotrophic), is located in multiple organelles including the cytosol, mitochondria, and endoplasmic reticulum. Different types of Trx have been suggested to interact with specific target proteins and thus regulate diverse processes (Bartsch et al., 2008; Marchand et al., 2010; Montrichard et al., 2009; Morisse et al., 2014). To summarize, the thiol groups within numerous proteins are prone to disulfide formation, which requires Trx to reverse the post-translational modification for proper function.

**Methods in Characterizing Thioredoxin Target Proteins**

Since the reduction of target proteins by Trx often leads to measurable changes in enzymatic activity of the targets, traditional biochemical analysis of the interaction between the substrates and the Trx relies heavily on the monitoring of substrate activity (Montrichard et al., 2009). For example, the activity of NADP-malate dehydrogenase, as measured by the absorbance at 340 nm, increased significantly when incubated with active Trx (Yoshida et al., 2015). For such *in vitro* reaction, Trx is typically heterogeneously expressed as tagged protein in *E.coli*. The relevance of the *in vitro* findings can be verified by investigation on the redox properties of the Trx-modulated proteins in *Trx* mutant plants (Yoshida et al., 2015). Biochemical and genetics tools have been extensively used in determining the functionality of Trx and target proteins, which was reviewed previously (Montrichard et al., 2009).

Other commonly used tools in defining PPI have also been applied in charactering Trx targets. For example, a Y2H identified the interaction between Trx $m2$ and voltage-dependent anion channel 3, which was further confirmed by pull-down assay and BiFC (Zhang et al., 2015). To pinpoint the regulatory Cys residues of the
substrates, site-directed mutagenesis can be performed to study the contribution of the individual Cys in redox regulation (Morisse et al., 2014).

**Proteomics in Characterizing Protein-Protein Interactions**

**Proteomics of MAPK-Substrate Interactions**

Methods employing mass spectrometry are excellent for identifying phosphorylation sites of MAPK substrates. Typically, substrates phosphorylated *in vitro* or *in vivo* are resolved by SDS-PAGE and the corresponding protein band is excised from the gel and digested with trypsin to release peptides. Phosphopeptides are typically enriched, and there are various methods available (Li et al., 2015b). Identification of phosphorylated residues sometimes can be ambiguous if multiple Ser or Thr residues are present in the peptide. Manual inspection of the MS/MS spectra is required to ensure fidelity of the phosphorylated residues. An alternative approach is mutagenesis of possible phosphorylation sites to Ala residues, coupled with monitoring of $^{32}$P-labeling of the mutant proteins.

The potential for identification and quantification of thousands of phosphopeptides in an unbiased manner makes MS-based phosphoproteomics a very attractive method to search for substrates of kinases. It has been used for identification of substrates of sucrose non-fermenting-1-related kinase (SnRK) 2.6 (Umezawa et al., 2013; Wang et al., 2013), chloroplast kinase (Reiland et al., 2009), thylakoid Ser/Thr protein kinase 8 (STN8) (Reiland et al., 2011), and tyrosine kinase (Mithoe et al., 2012). The first use of phosphoproteomics in plant MAPK substrate screening was reported in 2010, resulting in 141 proteins identified as possible MPK3/6 substrates (Hoehenwarter et al., 2013). In this approach, the phosphoproteomes of plants engineered to express little to no active kinase and of plants expressing wild type or hyperactive kinases are
quantitatively compared. Proteins with a significant difference in phosphorylation level are thus considered to be putative substrates of the kinase in question. An important feature of MAPKs is their low basal activity (Meng and Zhang, 2013; Li, et al., 2015a), making activation of the MAPK essential for initiation of downstream phosphorylation. In addition, selective enrichment of phosphoproteins and/or phosphopeptides is key for successful identification and quantitation because of the low abundance and substoichiometry of phosphoproteins. In another approach, enriched phosphopeptides are treated with a phosphatase to remove the phosphorylation modifications, and the resulting peptides were incubated with a kinase. Peptides with newly-formed phosphorylation represent candidate substrates (Xue et al., 2012). However, it is not certain that a particular phosphatase will be able to remove all phosphate groups on proteins in a cellular extract. In addition, a severe limitation lies in that the direct phosphorylation of the MAPK substrates and downstream indirect phosphorylation changes triggered by the activated MAPK cascades cannot be differentiated. This will inherently result in a false positive identification of MAPK substrates, making additional validation necessary.

Due to the rapid improvement in sensitivity and accuracy in mass spectrometry, affinity-purification coupled with mass spectrometry (AP-MS) is gaining popularity as a strategy for characterizing plant PPI (Dedecker et al., 2015). Typically, the protein of interest is expressed in fusion with a tag such as FLAG or GFP in stable transgenic plants, and the interacting complex is immunoprecipitated, followed by trypsin digestion and liquid chromatography MS/MS identification (Fabregas et al., 2013; Fujiwara et al., 2014). Tandem affinity tags have also been employed, providing a more stringent
purification of the *bone fide* interacting partners (Heijde et al., 2013). Application of this technique in MAPK substrate identification has not yet been reported. Given the transient nature of kinase-substrate interaction, cross-linking of the unstable complex may be required. Moreover, this approach may capture direct or indirect interacting partners. Therefore, additional evidence is needed to prove the authenticity of a putative kinase substrate. Once a putative substrate is identified, it can be asked: what are the interaction domains? What happens to the substrate when it is phosphorylated? What downstream event does it mediate? To answer these questions, genetic and biochemical studies will shed new light. Genetic epistasis analysis would validate the functional order of the MAPK-substrate in a specific physiological context, while detailed characterization of the physical interaction within the kinase-substrate pair (see Box 1 in Appendix A for available methods) is required to understand the basis of the interaction.

**Proteomics in Identifying Thioredoxin Target Proteins**

Several proteomics methods have been developed in efforts to identify Trx targets. Initially, the active Trx was incubated with plant protein extracts to allow thiol disulfide exchange reaction. The newly formed sulfhydryl groups was then irreversibly labeled with thiol-specific probes such as monobromobimane. Fluorescent probes enable the visualization of the potential target proteins when the reaction mixture was separated on either SDS-PAGE or two-dimensional-gels (Yano et al., 2001). Later on, an isotope-coded affinity tag has been used to quantitatively compare the redox status of Cys residue-containing peptides, thus providing a way to determine the reaction between Trx and target proteins.

Another way to identify Trx targets employs the reaction mechanism between Trx and the substrates. The first Cys residue in the WCGPC reaction center attacks the
disulfide bond of the substrate, creating a bridge between them. Then the second Cys residue breaks the heterodisulfide intermediate, releasing the reduced substrate from Trx. Thus, mutation of the the second Cys residue results in the formation of Trx-substrate complex, which can be pulled down and then disrupted by strong reducing reagents such as dithiothreitol. The isolated Trx target proteins can be analyzed by proteomics (Montrichard et al., 2009).
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Figure 1-1. Methodology in MAPK-substrate identification. A) General workflow in defining a MAPK-substrate mediated signaling in plants. Activation of MAPKs are triggered by upstream signals such as pathogen-associated molecular pattern flg22 and drought-induced plant hormone abscisic acid (ABA). Activated MAPKs interact and phosphorylate downstream substrates, leading to band upshift in a mobility assay. The phosphorylation site(s) are mapped and mutated to study the effect of phosphorylation on substrate properties. Methods listed in B are available to validate the physical interaction between MAPKs and substrates. Physiological roles of the interaction is determined using genetics and biochemical tools. Tests are designed to answer questions such as the specificity of the interaction, role of individual domain or site in interaction and activity, consequences of altered signaling aptitude. B) Strategies in MAPK-substrate identification and validation. Large scale methods for identifying MAPK substrates are listed in the left panel, and approaches in looking for a responsible MAPK for a substrates are listed in the right panel. Strategies for validating a MAPK-substrate interaction are listed in the middle panel.
Figure 1-2. Proposed model for activation of MAPK cascades. It is common that one MAPK cascade mediate numerous cellular responses. How is the signal specificity controlled in MAPK cascade activation and response? The MAPK activity is kept at basal level under normal plant growth conditions. MAPK cascades are activated by various upstream stimuli, such as developmental signals, biotic and abiotic stresses. The mechanism of MAPK cascades activation is largely unknown. However, different upstream signals are specifically transduced through at least binding of the terminal MAPK to distinct substrates, regulating an array of biological processes. Substrates are modulated by MAPKs in the following know ways: either increase or decrease in protein stability, activation or inhibition of enzymatic activity, re-localize into cellular compartment, and changes in the binding pattern of the substrates to other molecules such as protein and DNA. Depending on the function of substrates, various downstream cellular responses may be triggered. However, the molecular mechanism determining the direction of MAPK activation by different signals is unclear. The general proposed model is shown on the left and an example of MPK4 is shown on the right panel, with circled P indicating phosphorylation.
CHAPTER 2
OXIDATION AND PHOSPHORYLATION OF MAP KINASE 4 CAUSES PROTEIN AGGREGATION

Background

A few MAPK cascades have been shown to be activated in response to biotic and abiotic stress’ (Cardinale et al., 2002; Kiegerl et al., 2000; Suarez-Rodriguez et al., 2007; Xiong and Yang, 2003). Stress is also known to induce production of reactive oxygen species (ROS), known as oxidative burst, early in the response. For example, the stress-induced phytohormone abscisic acid (ABA) promotes stomatal closure, and the ROS burst plays a critical role in guard cell ABA signaling (Kim et al., 2010; Kollist et al., 2014). Furthermore, mounting evidence suggests that ROS signaling and MAP kinase activation are strongly intertwined in plants (Meng and Zhang, 2013). Previous investigations indicated that ROS may act upstream of the stress-activated MPK cascades (Opdenakker et al., 2012; Pitzschke and Hirt, 2006). However, evidence also showed that MAPK cascades regulate the expression of respiratory burst oxidase homologs (RBOHs), which are responsible for ROS production. For example, MEK in Nicotiana benthamiana is involved in the regulation of RBOH B at the mRNA level (Yoshioka et al., 2003). In addition, MPK5 in Zea mays is required for activation of NADPH oxidase (Lin et al., 2009).

Extensive studies have shown that MEKK1-MKK1/2-MPK4 pathways in Arabidopsis and tobacco are responsive to stress and plays an important role in ROS

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signaling (Brodersen et al., 2006; Droillard et al., 2004; Gomi et al., 2005; Petersen et al., 2000; Suarez-Rodriguez et al., 2007). A knock out mutant of the terminal kinase of this cascade, mpk4, shows constitutively active systemic acquired resistance (SAR), with high accumulation of salicylic acid (SA) and pathogenesis-related (PR) genes. The phenotypes of knock out mutants of the upstream kinases, mekk1/2 and mekk1, were similar to that of mpk4 (Gao et al., 2008; Ichimura et al., 2006; Nakagami et al., 2006; Pitzschke et al., 2009a; Su et al., 2007). Additionally, ROS metabolism and signaling components were disturbed similarly in the three mutants (Pitzschke et al., 2009a). For example, transcripts of major ROS-scavenging genes were reduced in the three mutants. Therefore, MEKK1-MKK1/2-MPK4 constitutes not only a pathway in response to stress (Ichimura et al., 2006; Suarez-Rodriguez et al., 2007), but also a major regulating module of ROS homeostasis (Nakagami et al., 2006; Pitzschke et al., 2009a).

MPK4 is highly expressed in guard cells and has been proposed to function in ABA induced stomatal closure (Berriri et al., 2012; Petersen et al., 2000; Zhu et al., 2009). However, activation of MPK4 in response to ABA in guard cells has not been observed. In addition, whether ROS as critical second messengers in guard cell signaling exert direct effect on MPK4 function is unknown. In this study, it was demonstrated that MPK4 is activated in response to H₂O₂ treatment, and active MPK4 triggers ROS production in leaves. To disentangle the connection between redox states of MPK4 and kinase function, the biochemical properties of recombinant MPK4 was characterized. We present evidence that phosphorylation of MPK4 causes protein aggregation in the presence of H₂O₂.
Materials and Methods

Plant Materials

*Brassica napus* plants were grown at 24 °C in light for 10 h and 20 °C in dark for 14 h. Two month-old plants were used to test responses to H₂O₂ and for guard cell isolation. *Nicotiana benthamiana* plants were grown at 24 °C under 16 h light and 8 h dark period. Six week-old plants were used for infiltration.

PCR Amplification of *B. napus* MPK4

Total RNA was extracted from two-month-old *B. napus* plants using an RNeasy mini kit following the manufacturer’s instructions (Qiagen, Valencia, CA, USA). One microgram total RNA was reverse-transcribed using a ProtoScript® II first strand cDNA synthesis kit (New England Biolabs, Beverly, MA, USA). MPK4 was amplified with the primers 5’-CGGATCCATGTCGGCGGAGAACTG-3’ and 5’-CCTCGAGTTACTGAGGATTGAACCTTG-3’. The forward and reverse primers contain a BamH I restriction endonuclease site and an XhoI site, respectively. PCR was performed using Phusion DNA Polymerase as follows: 98°C for 30 s (1 cycle); 98°C for 10 s, 55°C for 30 s, and 72°C for 30s (30 cycles); and 72°C for 10 min (1 cycle). The PCR product was cloned into a pSC-B vector (Stratagenem, La Jolla, CA, USA) and verified by sequencing.

In-gel Kinase Assay

Fully expanded leaves from two-month-old *B. napus* were sprayed with 5 mM H₂O₂, harvested at 1, 5, 10 and 30 minutes after the treatment, and frozen in liquid nitrogen immediately. Total protein was extracted in extraction buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 6 mM β-mercaptoethanol, 0.5 mM phenylmethysulfonyl fluoride, 10 mM Na₃VO₄, 10 mM NaF and protease inhibitor cocktail from Thermo Scientific).
After centrifugation at 20,000 g for 30 min, supernatants were transferred to new tubes and the concentration of protein was measured using a Bradford protein assay kit (Bio-Rad Laboratories, Los Angeles, CA, USA). The in-gel kinase assay was done as previously described with slight modification (Zhang and Klessig, 1997). Briefly, 25 μg protein aliquots were separated on 10% SDS-polyacrylamide gel that contained 0.5 mg/mL MPK4 substrate 1 (MKS1) as substrate. SDS was removed with three changes of washing buffer (25 mM Tris, pH 7.5, 0.5 mM DTT, 0.1 mM Na₃VO₄, 5 mM NaF, 0.5 mg/mL BSA, and 0.1% Triton X-100), and then the protein was renatured in renaturing buffer (25 mM Tris, pH 7.5, 1 mM DTT, 0.1 mM Na₃VO₄, and 5 mM NaF) for overnight. For the kinase reaction, the gel was incubated in 30 mL reaction buffer (25 mM Tris, pH 7.5, 2 mM EGTA, 12 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT, and 0.1 mM Na₃VO₄, 200 nM ATP, 50 μCi γ⁻³²P ATP) for 1 h. The gel was then washed extensively in 5% trichloroacetic acid and 1% sodium pyrophosphate. After washing, the gel was air-dried and then exposed to X-film in a -80 °C freezer.

**Protein Gel Blot**

Guard cell protoplast isolation was performed as previously described (Zhu et al., 2009). The protoplasts were incubated in basic solution for 1 h before ABA treatment. SDS sampling buffer was added to the treated protoplasts and the sample was boiled for 5 min for protein extraction. Protein samples were separated by 12% SDS-PAGE, transferred to nitrocellulose membrane and incubated with phospho-p44/42 antibody (Cell Signaling Technology, Beverly, MA, USA). Anti-rabbit IgG conjugated to horseradish peroxidase (Pierce, Rockford, IL USA) was used as secondary antibody. Protein signals were detected using ECL substrates (Thermo Scientific, Rockford, IL, USA).
Recombinant BnMPK4 Expression, Purification, and Identification

The open reading frame of *BnMPK4* (Accession number: HM563041) was subcloned as *BamH I*-Xhol fragment into the pET28a vector. The plasmid pET28-*BnMPK4* construct was transformed into *E.coli* strain BL21 (DE3). To generate *BnMPK4*<sup>C6A</sup> and *BnMPK4*<sup>C232A</sup> mutants, a site-directed mutagenesis method was used according to the manufacture’s guide (Stratagene, La Jolla, CA, USA). The following primers were used to amplify pET28-*BnMPK4* plasmid: 5’-

ATGTCGGCGGAGAACGCATTCGGCGGTGGCGGT-3’ and 5’-

ACCGCCACCACCGATCGTTCTCCGCGGAT-3’ for *BnMPK4*<sup>C6A</sup>, 5’-

TCATTGGTTTCACCAGAATAGCAACCACGACCAGATCGT-3’ and 5’-

CGATATCTGTTCTGGTGGTCTATTCTCGGTGAACATGA-3’ for *BnMPK4*<sup>C232A</sup>. The sequences of the two *BnMPK4* mutants were verified by sequencing.

Bacteria harboring the *BnMPK4* constructs were cultured with 50 μg/ml kanamycin to an OD<sub>600</sub> of 0.6, and the expression of 6×His-tagged BnMPK4 protein was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Recombinant protein was purified in a cold room using a PrepEase (Affymetrix Inc., Santa Clara, CA, USA) histidine-tagged protein purification kit following the manufacturer’s instructions. Briefly, the bacteria pellet was resuspended in 50 mM NaH<sub>2</sub>PO<sub>4</sub>/ Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl (pH 8.0) and sonicated, and the soluble supernatant was applied to the Ni-TED resin column. The BnMPK4 protein was eluted with 50 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, 250mM imidazole (pH 8.0) and dialyzed against 50 mM Tris-HCl (pH 7.5), 0.5 mM DTT. For subsequent treatment with H<sub>2</sub>O<sub>2</sub>, DTT was removed using Amicon ultra centrifugal filters with a 3 kDa cutoff in a cold room.
A similar procedure was used to purify BnMKS1 (MPK4 substrate 1 from *B. napus*). Briefly, the coding region of *BnMKS1* was amplified by PCR from *B. napus* leaves cDNA with the primers 5'-ATGGATCCGTCGGAGTCTTTC-3' and 5'-ATCCCAAATATGACTGAACAAATCAG-3'. The MKS1 fragment was then cloned into pET28a vector and verified by sequencing. The fusion protein was then expressed in *E. coli* as described above.

The identity of the purified recombinant protein was confirmed using mass spectrometry as described (Chen, 2006). Briefly, the band excised from an SDS-PAGE gel was subjected to in-gel digestion in 50 mM NH₄HCO₃ containing 6 mg/mL modified trypsin (Promega, Madison, WI, USA). The resulting peptides were extracted and desalted by ZipTip. The protein digest was injected directly into ABI QSTAR Elite (AB Sciex Inc., Redwood City, CA, USA). The generated MS/MS spectra were searched against a NCBI green plant database using Mascot (Matrix Science Inc., London, UK).

**Transient Expression of BnMPK4 in *N. benthamiana***

To make a kinase-inactive (IN) control, the invariant Lys at position 72 was mutated to Ala (MPK4<sup>IN</sup>). In addition, a constitutively active (CA) version of MPK4 (MPK4<sup>CA</sup>) was created by mutation of residues Asp198 and Glu202 to Glu and Ala, respectively, according to a previous report (Berriri et al., 2012). The mutants were made by the site-directed mutagenesis procedure described above. cDNA fragments of wild type MPK4 (MPK4<sup>WT</sup>), MPK4<sup>IN</sup>, and MPK4<sup>CA</sup> were cloned into pCAMBIA1300FLAG vector. The binary vectors were then transformed into *Agrobacterium tumefaciens* strain C58C1 by electroporation and confirmed by PCR. For transient expression, *Agrobacterium* harboring different constructs were cultured overnight (LB medium with 50 mg/L kanamycin and 25 mg/L rifampicin) to reach an OD<sub>600</sub> of 0.6. The bacteria were
then pelleted and suspended in an infiltration solution (50 mM MES, pH5.6, 10 mM MgCl2 and 150 µM acetosyringone) to OD600 of 0.4, and induced at 28 °C overnight. Activated bacteria were infiltrated into 7-week-old *N.benthamiana* leaves with a needleless syringe (Wydro et al., 2006). Two days post infiltration (dpi), leaves were detached for H2O2 detection (ThordalChristensen et al., 1997).

**H2O2 and DTT Treatment of Recombinant BnMPK4**

After DTT removal using Amicon 3 kDa cutoff filters, purified BnMPK4 was treated with 0.5 mM or 1 mM H2O2 for 20 minutes, and then treated with 10 mM DTT for additional 20 minutes at room temperature. The protein samples were then separated on non-reducing SDS gels followed by Coomassie staining or subjected to kinase assay.

**In vitro BnMPK4 Kinase Assay**

Protein kinase assays were performed as previously described (Droillard et al., 2004). Briefly, 2 µg of purified protein were mixed with kinase reaction buffer (25 mM Tris, pH 7.4, 10 mM MnCl2, 10 µM ATP and 5 µCi of γ-32P ATP). The reaction was carried out at 30 °C for 30 min. SDS loading buffer was added to stop the reaction, and proteins were separated by SDS-PAGE. For redox experiments, non-reducing SDS loading buffer and gels were used. The gel was stained with Coomassie Brilliant Blue (CBB) and destained with 50% methanol and 10% acetic acid, and then with 5% methanol and 7% acetic acid. The gel was dried and kinase activity was determined using autoradiography.

For autophosphorylation, the kinase activity assay was carried out using different amounts of protein ranging from 0.1 to 6 µg. Reactions were performed at 30 °C for 10 min and then stopped by adding the SDS loading buffer. Samples were then separated
using SDS-PAGE. Individual BnMPK4 bands were excised and subjected to radioactivity counting in a Beckman liquid scintillation counter (Beckman Coulter, Brea, CA, USA).

**Phosphorylation Site Mapping of BnMPK4 and BnMKS1**

To map the phosphorylation sites of BnMPK4 and BnMKS1, an *in vitro* kinase reaction was carried out as described previously (Huang et al., 2000). The reaction mixture was resolved by SDS-PAGE, and the desired protein bands were excised and trypsin digested. The in-gel digested peptides were lyophilized and phosphopeptides were enriched by using ZrO$_2$ tips (Glygen, Columbia, MD, USA). Phosphopeptides were separated on a nanoflow C18 column (Dionex, Pittsburg, PA, USA) and analyzed using a LTQ Orbitrap-XL mass spectrometer (ThermoFisher, Bremen, Germany) (Avila et al., 2012).

**Results**

**Isolation and Sequence Analysis of *BnMPK4***

The *BnMPK4* gene was isolated from *Brassica napus* leaf cDNA. The gene encodes a protein of 373 amino acids with calculated molecular mass of 42.4 kDa and pI of 5.8. The deduced amino acid sequence of MPK4 shows typical structural features of MAP kinase B group. It contains all 12 conserved kinase subdomains, with the TEY residues located in the activation loop between VII and VIII (Figure 2-1). The common docking (CD) domain was located at the C terminus and may be involved in interaction with other proteins. Sequence comparison showed that BnMPK4 shares 94.95% identity with AtMPK4. The close relationship between BnMPK4 and AtMPK4 was also observed in the phylogenetic analysis of orthologous plant MPK4s (Figure 2-1). Interestingly, we observed differences of three amino acids among the MPK4s from three *B. napus*.
cultivars: *Global*, *Zhongshuang* and *Huyou 15*. The amino acids were located in a variable sequence region between the conserved kinase domains (See Figure 2-1).

**BnMPK4 Is Activated by H$_2$O$_2$**

To determine whether BnMPK4 is responsive to H$_2$O$_2$ *in planta*, an in-gel kinase assay was performed using crude protein extracts from two-week-old *B. napus* plants treated with 5 mM H$_2$O$_2$ for different time length. Recombinant *B. napus* MKS1, ortholog of the specific MPK4 substrate identified in Arabidopsis (Andreasson et al., 2005), was embedded in the gel as a substrate. A single labeled band was observed on the X-film at around 44 kDa, the predicted mass of MPK4. A previous study also showed that closely related MPKs such as MPK3 and MPK6 do not phosphorylate MKS1 (Berriri et al., 2012). As shown in Figure 2-2A, MPK4 activity responded rapidly to H$_2$O$_2$ at 1 min, and peaked at 5-10 min. The signal dropped to the basal level at 30 min, indicating the H$_2$O$_2$ triggered MAP kinase signaling is a fast event.

Activation of MAP kinases plays an important role in guard cell signaling, and ROS production is an essential intermediate step in ABA-induced stomatal closure. To test whether in guard cells MPK4 is also downstream of ABA, guard cell protoplasts were isolated from *B. napus* leaves and then treated with 50 µM ABA. The active form of MPK4 was detected using phospho-p44/42, an antibody that recognizes three phosphorylated MPKs in plants, MPK3, MPK4 and MPK6 (Massoud et al., 2012). In SDS-PAGE MPK4 is the fastest migrating protein of the three. The results revealed that MPK4 is activated at 10 min after treatment in isolated guard cell protoplasts (Figure 2-2B). Based on the above results of H$_2$O$_2$ and ABA experiments, BnMPK4 acts downstream of ABA and H$_2$O$_2$. The MPK4 activity peaked significantly earlier in H$_2$O$_2$
treatment compared to ABA treatment, supporting the idea that ROS is produced in
downstream of ABA.

**Constitutively Active BnMPK4 Promotes ROS Production and Cell Death**

ROS-triggered MAP kinase cascades play important roles in plant signaling
(Jiang et al., 2008; Opdenakker et al., 2012). However, evidence also showed that the
MAP kinase pathways could lead to ROS burst (Pitzschke and Hirt, 2009). To explore
the possible role of MPK4 in ROS production *in planta*, we transiently expressed wild
type and mutated BnMPK4s in *N. benthamiana* leaves. All three constructs had a
3×FLAG tag fused to the C terminus of BnMPK4 (Figure 2-3A). Western blots
developed with anti-FLAG showed similar expression levels of the three MPK4
constructs in *N. benthamiana* leaves, while no expression was detected in the vector
control (Figure 2-3B). It was reported that overexpression of constitutively active MAP
kinase cascade components including MKK4-MPK4, MKK5-MPK1, MKK-MPK4, MKK7-
MKP1, and MKK9-MPK3, and MKK0-MPK10, triggered cell death in *N. benthamiana*
leaves (Popescu et al., 2009). No cell death was detected when wild type MPK4 was
expressed in *N. benthamiana* leaves (Figure 2-3C). Similarly, expression of the inactive
MPK4 mutant did not trigger cell death either. In contrast, cell death was observed 2
days post-infiltration (dpi) in leaf sections infiltrated with MPK4<sup>CA</sup>. These data
demonstrate that constitutive kinase activity of MPK4 is necessary for the cell death
phenotype. To determine whether ROS was produced in this process, leaves were
stained with 3,3'-diaminobenzidine (DAB). As shown in Figure 2-3D, cell death was
accompanied by accumulation of H<sub>2</sub>O<sub>2</sub>.
Recombinant BnMPK4 From *E. coli* is an Active Kinase

The findings that MPK4 acts both downstream and upstream of ROS production prompted us to examine this kinase biochemically and to ask if it is regulated by ROS. Purified bacterially-expressed BnMPK4-His was resolved on SDS-PAGE (Figure 2-4A) and its identity confirmed by mass spectrometry (Figure 2-4B). Autophosphorylation assays performed with different divalent cations showed no activity in the presence of Ca$^{2+}$ but strong autophosphorylation of MPK4 was observed with Mn$^{2+}$ relative to Mg$^{2+}$ (Figure 2-5A). Thus, all subsequent assays were performed with Mn$^{2+}$.

Transphosphorylation activity was examined with the well characterized substrate MKS1 (Andreasson et al., 2005). Figure 2-5B shows that BnMPK4 phosphorylated BnMKS1. These data demonstrate that recombinant BnMPK4 is active in the absence of the upstream kinases in the cascade *in vitro*.

Identification of Phosphorylation Sites in BnMPK4 Using Mass Spectrometry

To understand the activation mechanism of BnMPK4, the phosphorylation sites were mapped. The autophosphorylation sites were identified by *in vitro* kinase assay and mass spectrometry analysis. Phosphorylation on Tyr203 was readily detectable. This is consistent with previous phosphoamino acid analysis showing that the Arabidopsis MPK4 autophosphorylated mainly on Tyr (Huang et al., 2000). In addition, Thr201 in the activation loop was also phosphorylated (Figure 2-6A and B). Thus, the self-activation of MPK4 *in vitro* is probably due to the dual phosphorylation on both Thr201 and Tyr203.

We also mapped the phosphorylation sites on MKS1. MAPKs usually phosphorylate serine or threonine residues followed by a proline. No Thr-Pro motifs are present in the MKS1 sequence. Among the 10 Ser-Pro motifs present in BnMKS1, two
sites were found to be phosphorylated. These two sites were also reported in earlier studies of *Arabidopsis* (Caspersen et al., 2007), suggesting they may be conserved MPK4 phosphorylation sites (Figure 2-6C).

**Oxidation and Phosphorylation of Leads to BnMPK4 Aggregation**

Our data show that H$_2$O$_2$ activates MPK4, and active MPK4 may promote ROS production. To investigate the direct effect of ROS on MAP kinase conformation and activity, recombinant BnMPK4 was subjected to redox treatment and kinase activity assay. Surprisingly, when treated with H$_2$O$_2$ and analyzed by non-reducing SDS-PAGE, the expected protein bands on SDS-PAGE were very faint. Instead, bands were observed in the loading well (Figure 2-7A, Lanes 6 and 7). We identified these bands as BnMPK4 using mass spectrometry. The electrophoretic mobility shift indicated aggregation of the protein. The aggregated kinase retained activity, showing that the catalytic sites were still functional (Figure 2-7B). To examine whether the aggregation was reversible, the H$_2$O$_2$-treated BnMPK4 was subjected to buffer exchange, treated with DTT and tested for kinase activity. Interestingly, DTT treatment reduced the aggregation and the BnMPK4 bands were observed at the expected size (Figure 2-7A, Lane 8 and 9), and the monomers also retained kinase activity (Figure 2-7B). Aggregation was observed in the untreated control protein, which could be due to the oxidation of the protein by air exposure.

To determine whether the aggregation was caused by H$_2$O$_2$-treatment or autophosphorylation, BnMPK4 treated with H$_2$O$_2$ and then DTT, as described above, was mixed with kinase reaction buffer without ATP and incubated at room temperature for 30 min to mimic the conditions of the kinase reaction. When resolved on SDS-PAGE, all the samples showed similar monomeric mobility patterns (Figure 2-7A, Lane
This suggests that the redox treatment alone did not change the conformation of recombinant BnMPK4, and phosphorylation of BnMPK4 in the absence of reducing reagent led to protein aggregation. In addition, when the proteins prepared as in Fig. 2-7A were run on a reducing SDS-PAGE gel, no aggregation was observed (Figure 2-8). This indicates that both oxidation and phosphorylation are necessary for aggregation to occur.

**Cysteine Involvement in the Aggregation During BnMPK4 Phosphorylation**

Formation of disulfide bonds between cysteine residues represents one mechanism for reversible protein aggregation (Zhu and Labuza, 2010). There are eight cysteines in BnMPK4. To ask whether oxidation of cysteines is responsible for the protein aggregation, we first evaluated possible disulfide bond connections within BnMPK4 using DiANNA, a web based tool for disulfide connectivity prediction (Ferre and Clote, 2005). The results showed that Cys6 and Cys232 had high potential for disulfide bond formation. To test the role of these cysteine residues in BnMPK4 aggregation, BnMPK4\textsubscript{C6A} and BnMPK4\textsubscript{C232A} were constructed. The mutant versions of the BnMPK4 were expressed in *E. coli* and then purified for kinase assays. Both mutants showed autophosphorylation and transphosphorylation activities (Figure 2-9A). BnMPK4\textsubscript{C6A} aggregated in the presence of 1mM H\textsubscript{2}O\textsubscript{2} in the kinase reaction buffer (Figure 2-9B, lane 1), but not with 10 mM DTT (Figure 2-9B, lane2). However, no protein aggregation was observed for BnMPK4\textsubscript{C232A} in the same experimental condition with or without H\textsubscript{2}O\textsubscript{2} (Figure 8B, lanes 3 and 4). These results suggest Cys232 may be involved in BnMPK4 aggregation when autophosphorylated in the presence of oxidants.
**Discussion**

In this study, we investigated the connection between ROS and BnMPK4. Our data are consistent with a regulatory model where ROS together with MPK4 play a role in stress signal transduction. In this model, stress induced ROS activate MPK4, and it in turn promotes ROS production. Thus, the stress signal is boosted to induce the proper cellular response. To terminate or attenuate the signal, a negative feedback mechanism is needed to bring the signaling system to a steady state. Possible mechanisms include a scavenger system to remove excess ROS, and/or a protein phosphatase to deactivate MPK4. Here our data suggests that phosphorylation of MPK4 in the presence of H$_2$O$_2$ causes protein aggregation without loss of activity, which could sequester the activity away from substrates, or possibly aggregated MPK4 may be subjected to protein degradation.

ROS are thought to be involved in activation of MPKs because stresses usually trigger simultaneously the ROS burst and MPK activation (Apel and Hirt, 2004; Pitzschke et al., 2009a; Pitzschke and Hirt, 2006). In addition, exogenous H$_2$O$_2$ is sufficient to activate MPKs (Kovtun et al., 2000; Xu et al., 2014). Our data show that spraying H$_2$O$_2$ on *B. napus* leaves activates MPK4. In addition, treatment of guard cell protoplasts with ABA, which promotes rapid ROS bursts (Desikan et al., 2004), also triggers MPK4 activation. These observations are consistent with previous findings that MPK4 is a stress-activated protein kinase (Berriri et al., 2012; Brodersen et al., 2006; Schweighofer et al., 2007). However, the underlying mechanism of how ROS activate MPK4 is still elusive. In mammalian cells, it has been proposed that the stress-induced ROS burst may inactivate MAP kinase phosphatases (MKPs), thus activating MAP kinase pathways (Son et al., 2011). Interestingly, a recent study showed that ROS may
not be necessary for MPK activation (Xu et al., 2014). For example, flg22, a bacterial derived pathogen-associated molecular pattern (PAMP), strongly promotes ROS burst and activates MPK3, MPK4 and MPK6. Nevertheless, in atrbohD mutant where no ROS burst was observed after flg22 treatment, the activation of MPKs is still normal. While it remains to be seen whether other stress-induced MPK activation is ROS dependent or not, it is possible that MAPK activation triggered by exogenous ROS could be an indirect effect of ROS application. Alternatively, another pathway that is independent of ROS functions parallelly to ROS pathway to activate MAPK cascades under stress conditions.

MPK4 is not only activated by ROS, but the kinase also functions in controlling ROS homeostasis (Pitzschke et al., 2009a). The mpk4 mutant accumulated higher level of H₂O₂ than wild type plants (Petersen et al., 2000), and an enhanced ROS burst was detected in mpk4 mutant in response to flg22 (Xu et al., 2014). In contrast, flg22-induced ROS burst is normal in mpk3 mpk6 double mutant (Xu et al., 2014). These findings highlight the essential role of MPK4 in ROS metabolism. We envisioned that ROS level should be abnormal in plants with a higher MPK4 activity. However, MAP kinase cascades are stringently controlled in plants, overexpression of a normal kinase may not enhance kinase activity in planta (Xu et al., 2014). Our observation that transient expression of a wild type MPK4 in N.benthamiana leaves does not trigger an ROS burst or cell death supports this idea. In contrast, transient expression of constitutively active MPK4 is associated with ROS burst and cell death. This is also in line with a previous report, in which MPK4 together with a constitutively active upstream kinase were coexpressed and cell death was observed (Popescu et al., 2009). An
unresolved issue is how enhanced MPK4 activity alters the ROS burst. RBOH is thought to be responsible for rapid ROS production in the early stage of the response, while metabolic imbalance in organelles such as chloroplasts may contribute to the sustained ROS formation (Apel and Hirt, 2004). In addition, regulation of RBOH D by MPK8 via direct phosphorylation was reported (Takahashi et al., 2011). Thus, it will be interesting to see whether BnMPK4 also affects RBOH activity.

Since ROS and MPK4 may constitute an amplification loop for the upstream signal, we reasoned that there might be redox regulation of MPK4 function. Thus more detailed biochemical studies were conducted in this study. We isolated the MPK4 gene from *B. naupus* and showed that the encoded protein is an active protein kinase (Figures 2-2, 2-3 and 2-5). Mass spectrometry data suggest that the recombinant protein autophosphorylates on the conserved Thr and Tyr in the activation loop. It was previously thought that MAPK is largely activated by an upstream kinase and autophosphorylation activity of most MAPK was lost during evolution (Tzarum et al., 2013). Interestingly, there is also convincing evidence showing that MAPK homologs in mammal cells are activated by autophosphorylation (Gills et al., 2007; Kim et al., 2005). MPK4 has shown to be dual-phosphorylation kinase (Huang et al, 2000; Figure 2-6 in this study) and phosphorylation on Tyr may occur through autophosphorylation. Therefore, autophosphorylation may represent a self-regulatory mechanism for MPK4 activation, while the phosphorylation by upstream kinases might be required for high MPK activity. Since MAPK cascades are tightly controlled and basal level of MAPK activity is typically low, it possible that lacking of an inhibitory regulator such as a phosphatase for MPK4 in *E.coli* is responsive for MPK4 self-activation. In such a
scenario, a phosphatase removes the Thr and/or Tyr phosphorylation that results from autophosphorylation to keep MPK4 in an inactive form. Upstream stimuli such as ABA and H$_2$O$_2$ abolish the inhibition of the phosphatase, thus releasing MPK4 as active kinase. This self-regulatory pathway could coexist with the canonical MAPK cascade, since the linear MEKK1-MKK1/MKK2-MPK4 cascade does not account for all the physiological role that MPK4 plays (Ichimura et al. 2006).

The auto-activated BnMPK4 is able to phosphorylate substrate protein BnMKS1 (Figure 2-5). MKS1 was identified as an in vitro MPK4 substrate in an earlier yeast two hybrid screening (Andreasson et al., 2005). Further studies confirmed that MPK4 interacts and phosphorylates MKS1 in Arabidopsis (Andreasson et al., 2005; Petersen et al., 2010). To map the phosphorylation sites, the purified recombinant AtMKS1 was phosphorylated by hemagglutinin (HA)-tagged AtMPK4 immunoprecipitated from Arabidopsis. Three phosphorylation sites, Ser72, Ser108, Ser120, were identified using mass spectrometry (Caspersen et al., 2007). Here we have identified two of the three phosphorylation sites in BnMKS1 phosphorylated by BnMPK4, suggesting MAP kinases in different species phosphorylate substrates on the conserved motifs.

The autophosphorylation and activation feature of recombinant MPK4 enable us to characterize the redox regulation of BnMPK4. Using in vitro kinase assay, we observed that in the presence of H$_2$O$_2$, phosphorylation of BnMPK4 caused protein aggregation. The aggregated protein retained autophosphorylation activity and could be converted into monomeric form by DTT. These results suggest that reversible modifications on certain amino acids took place during phosphorylation in the presence of H$_2$O$_2$. Because mutation of Cys232 abolished the aggregation in the presence of
H$_2$O$_2$, it is likely that cysteine oxidations including disulfide bond formation contributes directly to the protein aggregation. Cys232 is located within the conserved subdomain IX, which forms an α-helix and helps to stabilize the catalytic loop (Hanks and Hunter, 1995). The fact that the C232A mutant retains kinase activity indicates that replacement of the Cys with Ala did not change the overall protein structure. Instead, cysteine modification is abolished in the mutant. Collectively, these results suggest a possible feedback link between cellular redox state and BnMPK4 activity. The importance of cysteine modification in redox status sensing and response has been suggested in guard cell ABA signaling (Zhu et al., 2014). In addition, a recent study showed that reversible cysteine modification plays a crucial role in converting aggregates peptides to functional peptide hormone (Anoop et al., 2014). Unraveling the in vivo cysteine modification and conformational change of MPK4 will be necessary to fully characterize the possible role of aggregation in MPK4 functioning.
Figure 2-1. Sequence analysis of BnMPK4. A) Protein sequences were aligned using Clustal X. The aligned data were used to generate phylogenetic tree using MEGA4.0 with bootstrap value of 500. Branch lengths indicate genetic distance. MPK4s of selected plant species and accession number are: Bn, Brassica napus (DQ206628 from cultivar Huyou 15, HM563041 from cultivar Global and EU581868 from cultivar Zhongshuang No.9); At, Arabidopsis thaliana (AAZ20637); Nt, Nicotiana tabacum (AE46985); Na, Nicotiana attenuata (HQ236013); Gh, Gossypium hirsutum (ADI52627); Sl, Solanum lycopersicum (ADK38705); Zm, Zea mays (BAA74733.1); Vv, Vitis vinifera (XP002276158). The asterisk denotes BnMPK4 used in this study. B) Sequence alignment of Brassica napus MPK4 from three cultivars with Arabidopsis thaliana MPK4. Note that there are three amino acid variations among the three cultivars, indicated by arrows. They are at position 115, 147 and 370. The 11 major conserved subdomains of a protein kinase are marked by Roman numerals. The dual phosphorylation motif (TEY) is shown by double dotted red lines, while a common docking (CD) motif at the C-terminus is indicated by a red line. Amino acids shaded in pink indicates sequence difference.
Figure 2-2. MPK4 is activated by H$_2$O$_2$ and ABA. A) MPK4 is activated by H$_2$O$_2$ in *B. napus* leaves. Proteins extracted from leaves treated with 5 mM H$_2$O$_2$ at different time points were separated on MKS1 embedded gel. After renaturing the proteins, in gel kinase reaction was performed. The gel was stained with Coomassie brilliant blue (CBB). Top panel, autoradiography signals to indicate MPK4 activity. Bottom panel, CBB staining of Rubisco to indicate protein loading. B) MPK4 is activated by ABA in *B. napus* guard cell protoplasts. Protein samples were prepared from isolated guard cell protoplasts. Samples were collected at indicated time after 50 µM ABA treatment and analyzed by western blot using a Phospho-p44 / 42 MAPK antibody. MPK4 is the fastest migrating band. Top panel, protein gel blot to indicate MPK4 activity. Bottom panel, Ponceau S staining to indicate protein loading.
Figure 2-3. Constitutive active MPK4 triggers ROS production and cell death in *N. benthamiana* leaves. A) Schematic representation of the T-DNA region of pCAMBIA1300S-MPK4s-3 x FLAG for transient expression in infiltrated *N. benthamiana* leaves. Red arrow, double CaMV 35S promoter; Green arrow, CaMV 35S promoter; Black boxes, Nos terminators; RB, T-DNA right border; LB, T-DNA left border. B) Protein gel assay to detect the expression of MPK4s in *N. benthamiana* leaves at 2 dpi. C) Cell death phenotype of a 2 days post infiltration (dpi) leaf infiltrated with MPK4CA. The right part of the leaf was infiltrated with the four constructs in the same pattern as in the left part. The spots are: upper left, vector control (VC); upper right, C58C1 harboring pCAMBIA1300S-MPK4WT-3 x FLAG; bottom left: C58C1 harboring pCAMBIA1300S-MPK4IN-3 x FLAG; bottom right, C58C1 harboring pCAMBIA1300S-MPK4CA-3 x FLAG. D) DAB staining of the leaf in C showing H2O2 accumulation in leave areas expressing MPK4CA.
Figure 2-4. Heterologous expression and identification of *B. napus* MPK4. A) Coomassie blue-stained polyacrylamide gel showing a single protein band at approximately 44 kDa, the predicted molecular weight of the recombinant MPK4 protein. B) MPK4 protein band was excised and identified using MS/MS (information dependent acquisition). The phosphorylation sites identified were underlined by double green lines.
Figure 2-5. BnMPK4 is an active kinase. A) Divalent cation requirement for MPK4 autophosphorylation activity. Reactions were carried out in the absence of exogenous substrate for 30 min at room temperature in presence of 10 mM MgCl₂, 10 mM MnCl₂ or 10 mM CaCl₂. Autoradiograph (upper panel) showing the amount of ³²P-phosphate incorporation, and Coomassie staining of the gels (lower panel) depicting equal protein loading. B) Autophosphorylation and substrate phosphorylation activities of MPK4. While MPK4 can phosphorylate its substrate MKS1, the addition of MKS1 can enhance the autophosphorylation of MPK4 (upper panel). The low panel showing protein loading by Coomassie staining.
Figure 2-6. Identification of BnMPK4 and BnMKS1 phosphorylation sites. A) and B) MS/MS spectra of SETDFMpTEYVVTR and SETDFMTEpYVVTR from BnMPK4 showing the phosphorylation sites mapped to the threonine (T) and tyrosine (Y) within the activation loop of BnMPK4. C) Alignment of BnMKS1 and AtMKS1. Red arrow heads indicate phosphorylation sites identified in both BnMKS1 and AtMKS1, and green arrow head indicate an additional phosphorylation site identified in AtMKS1.
Figure 2-7. Phosphorylation of oxidized MPK4 causes protein aggregation. A) Coomassie blue-stained non-reducing SDS gels used for kinase assay of MPK4 in the presence of H$_2$O$_2$ and DTT. Proteins were treated with the indicated amounts of H$_2$O$_2$ and followed by incubation with (lanes 4-5 and 8-9) or without 10 mM DTT (lanes 1-3 and 6-7). Lanes 1-5 contain protein that was not subsequently subjected to the kinase reaction. Proteins in lanes 6-9 were incubated with ATP in the kinase assay. B) Autoradiogram of MPK4 that was oxidized with H$_2$O$_2$, then treated with or without DTT, then subjected to the kinase assay. Aggregated MPK4 retained kinase activity as shown by the radioactivity. The black arrows indicate the position of monomeric MPK4 on the gels.
Figure 2-8. Phosphorylation-induced protein aggregation can be reversed by DTT. The condition of redox treatment and kinase assay were the same as in Figure 6A. Instead of non-reducing buffer, 50 mM dithiothreitol (DTT) was used in the SDS sample buffer. Note that the protein aggregates could be reversed completely.
Figure 2-9. The role of cysteines in the aggregation of recombinant BnMPK4 protein. A) Kinase assay of the cysteine mutants of MPK4\textsuperscript{C6A} and MPK4\textsuperscript{C232A}. The mutant proteins were expressed in \textit{E. coli} and purified for kinase assay. Both autophosphorylation and transphosphorylation of BnMKS1 were assayed. The upper panel shows the autoradiograph and the lower panel shows the Coomassie staining of the same gel. B) C232A mutant abolished the protein aggregation. MPK4\textsuperscript{C6A} and MPK4\textsuperscript{C232A} proteins were treated with 1 mM H\textsubscript{2}O\textsubscript{2} at room temperature for 15 min, aliquot of the treated protein samples were then treated with 10 mM DTT (lanes 2 and 4). The redox treated protein samples were then allowed for kinase reaction, followed by SDS-PAGE and autoradiography.
CHAPTER 3
ECTOPIC EXPRESSION REVEALS PHOSPHORYLATION DYNAMICS AND INTERACTING PROTEINS OF MPK4 IN PLANT IMMUNITY

Background

MPK cascades are highly conserved and essential signaling components in plant immune responses (Asai et al., 2002; Meng and Zhang, 2013; Pitzschke et al., 2009b). MPK4, one of the most extensively studied MAPKs, plays multiple roles in at least two layers of plant innate immunity. In the first tier, pathogen-associated molecular patterns (PAMPs) such as the bacterial flg22 peptide are recognized by plant cell pattern recognition receptors (PRRs) such as the flagellin sensing 2 (FLS2) (Chinchilla et al., 2006). Recognition of flg22 triggers the activation of two MAPK cascades, one of which is MEKK1-MKK1/MKK2-MPK4 (Bethke et al., 2009; Li et al., 2015; Suarez-Rodriguez et al., 2007). As a component of this PAMP-triggered immunity (PTI), MPK4 has been suspected to regulate transcription factor activity and pathogenesis-related (PR) gene expression, as well as to maintain ROS homeostasis (Gao et al., 2008; Petersen et al., 2000; Pitzschke et al., 2009a; Qiu et al., 2008a). However, successful pathogens secrete effectors in order to suppress PTI and facilitate invasion. Plants, in turn, evolved resistance (R) proteins to initiate effector-triggered immunity (ETI) as a second layer of defense. ETI is often associated with elevated salicylic acid (SA) levels and hypersensitive response (HR) characterized by localized cell death (Cui et al., 2015). MPK4 is essential in SA accumulation, as demonstrated by the high level of SA in the Arabidopsis mpk4 mutant (Petersen et al., 2000). Transient expression of a constitutively active (CA) MPK4 in Nicotiana benthamiana leaves also triggers HR-mimic cell death (Zhang et al., 2015b). In addition, MPK4 negatively regulates the activity of the R protein SUMM2 (suppressor of mkk1 mkk2) (Zhang et al., 2012).
Thus, MPK4 functions at multiple levels in plant immune responses. However, the underlying mechanism remains unclear due to our limited knowledge of MPK4 interacting partners.

Manipulating the balancing of jasmonic acid (JA) and SA signaling is an important feature of MPK4 in plant immunity. Constitutive activation of SA-dependent systemic acquired resistance was observed in *mpk4*, with elevated PR gene expression (Petersen et al., 2000). The *mpk4* mutant also demonstrated impaired JA signaling, since the expression of JA-induced genes such as *PDF1.2* is blocked in *mpk4*. Thus, *mpk4* exhibits enhanced resistance to the virulent bacterial pathogen *Pseudomonas syringae pv. tomato* DC3000 (Petersen et al., 2000) and increased susceptibility to necrotrophic fungi *Alternaria brassicicola* (Brodersen et al., 2006). The former effect is considered to be a consequence of elevated JA response, while the latter results from impaired JA signaling.

The first functional study of MPK4 in species other than Arabidopsis also showed that MPK4 is required for JA signaling in *Nicotiana tabacum*. The MPK4-silenced tobacco plants showed undetectable kinase activity and displayed a dwarf phenotype. In addition, they accumulated significantly higher levels of SA and showed impaired stomatal closure, thus demonstrating high sensitivity to ozone stress, which induces the biosynthesis of SA (Gomi et al., 2005). Similarly, silencing MPK4 in *Nicotiana attenuata* led to impaired stomatal closure in response to ABA, H$_2$O$_2$ and dark (Hettenhausen et al., 2012). The function of MPK4 in guard cells is of particular interest, due to the fact that *MPK4* has been shown to be highly expressed in guard cells and that guard cells constitute the front line of plant defense (Berriri et al., 2012; Petersen et al., 2000).
Apart from regulation in defense response, MPK4 is also involved in cytokinesis (Kosetsu et al., 2010), microtubule organization (Beck et al., 2010) and mRNA decay (Roux et al., 2015). Due to this multifunctional nature of MPK4, the \textit{mpk4} mutant in Arabidopsis shows pleiotropic effects. The \textit{mpk4} mutant showed impaired root cell plate formation (Kosetsu et al., 2010) and microtubule organization (Beck et al., 2010), resulting in retarded root development and improper branching patterns of root hairs. Genetics approaches had been used to identify key players in MPK4-controlled processes by screening mutants that rescue the \textit{mpk4} phenotypes. For example, both enhanced disease susceptibility 1 (EDS1) and phytoalexin deficient 4 (PAD4) had been shown to be positive regulators in SA-dependent immune response downstream of MPK4 (Brodersen et al., 2006). Similarly, microtubule-associated protein 65 (MAP65) had been identified as a putative target of MPK4 in microtubule formation. However, an \textit{in vitro} kinase assay showed that EDS1 is not a direct substrate of MPK4 (Brodersen et al., 2006), and direct evidence showing interaction of MPK4 and MAP65 is lacking (Sasabe et al., 2011). These studies suggest that MPK4 interacts with as-of-yet unidentified proteins to mediate different physiological activities.

Identification of interacting protein partners is a critical step in elucidating the basis of multifunctional kinases such as MPK4. Several experimental approaches, including yeast two hybrid (Andreasson et al., 2005) and protein microarray (Feilner et al., 2005), had been developed to screen for MAPK targets. Recently, advancements in liquid chromatography–tandem mass spectrometry (LC-MS/MS) make it a robust tool to this end (Fabregas et al., 2013; Wang et al., 2013). When coupled with affinity purification (AP) of the protein of interest \textit{in vivo}, the AP-MS strategy not only identifies
putative bone fide interacting proteins, but also localizes specific phosphorylation sites within a kinase substrate. To gain a better understanding of the role of MPK4 in plant immunity, transgenic Arabidopsis plants carrying a FLAG-tagged MPK4 from Brassica napus was created. The BnMPK4 has been demonstrated to be activated by H$_2$O$_2$, whose production is further promoted by MPK4 activity. This study showed that ectopic overexpression of BnMPK4 enhances plant resistance to Pst DC3000, possibly through suppression of JA signaling and promotion of ROS production and thus stomatal closure. In addition, activation and phosphorylation dynamics of MPK4 triggered by flg22 were demonstrated. Using AP-MS analysis of the transgenic plants, putative MPK4 interacting proteins were identified, providing potential links between MPK4 and a diverse array of pathways in which the kinase participates.

**Materials and Methods**

**Plant Materials**

*Arabidopsis thaliana* Columbia (Col-0) was used for this study. The seeds were surface-sterilized using 50% bleach for 12 min and then were sown on $\frac{1}{2}$ Murashige and Skoog (MS) medium supplemented with 1× MS vitamins (Caisson, North Logan, UT, USA). After treatment at 4 °C in the dark for 2 days, the seeds were allowed to germinate in a growth chamber at 22 °C under a 16 h light/8 h dark photoperiod cycle. To create transgenic plants, the seedlings were transferred into soil (Metromix, Seba Beach, AB, Canada) and were grown under the same conditions used for germination. The floral dip method (Clough and Bent, 1998) was used for transformation with agrobacteria carrying the MPK4 construct described previously (Zhang et al., 2015b). The T$_0$ seeds were selected on $\frac{1}{2}$ MS medium supplemented with 50 mg/mL hygromycin. The T$_3$ plants were used for the physiological and proteomics studies.
**Pathogen Infection**

Infection with the bacterial strain *Pst* DC3000 was carried out by infiltrating four-week-old plant leaves (Ding et al., 2015). The bacteria was cultured in King’s B medium to OD\(_{600}\) of 0.4 and then pelleted by centrifugation at 3,000 rpm at room temperature for 1 min. The collected bacteria was resuspended and then diluted to OD\(_{600}\) of 0.0001 with 10 mM MgCl\(_2\). A needleless 1 mL syringe was used to inoculate the leaves. Three days after inoculation, the infected leaves were cut with a hole puncher and ground by a pestle in 1.5 mL tubes containing 10 mM MgCl\(_2\). The bacteria cell extract was diluted 400 times and plated on King’s B medium for colony unit formation counting.

**Phytohormone Measurement**

Metabolites were extracted from two-week old seedlings grown on ½ MS plates. The internal standard lidocaine was added to 100 mg of fresh-weighted plants, and the samples were ground into fine powder. Buffer 1 (80% methanol), Buffer 2 (37.5% acetic acid, 37.5% isopropanol) and Buffer 3 (50% acetic acid) were used sequentially for extraction at 4 °C on a rotating system for 15 min / buffer. Extracted metabolites were dried using a vacuum concentrator and then measured using a targeted multiple reaction monitoring (MRM) method (Misra et al., 2015).

**Stomatal Movement and ROS Measurement**

To measure the stomatal response to flg22, leaves from four-week-old plants were detached and then blended in tap water for 20 s. The epidermal strips were collected by filtering the blended mixture through a mesh with a pore size of 100 µm. To promote stomatal opening, the samples were incubated in a stomatal opening buffer (SOB, 10 mM MES-Tris, pH 6.0, 30 mM KCl, and 1mM CaCl\(_2\)) for 2 h under light (150 μE.m\(^{-2}\).s\(^{-1}\)). To induce stomatal closure, 10 µM flg22 were added to the buffer. Stomatal
Apertures were measured 1 h after treatment using a DM6000B light microscope (Leica, Buffalo Grove, IL USA) and Image J (http://imagej.nih.gov/ij/).

The ROS level was measured using the non-fluorescent dye 2’, 7’-dichlorofluorescin diacetate (H$_2$DCF-DA, Sigma-Aldrich, Saint Louis, MO, USA) (Zhu et al., 2016). The dye was loaded into the guard cells after incubation in the SOB, and the excess dye was removed by washing three times with SOB. After flg22 treatment for 15 min, the production of ROS was imaged by the DM6000B microscope with a Leica Kubler Codix external light source. The images were analyzed with Image J for quantification.

**Protein Extraction and Western Blot**

Total proteins were extracted from two-week-old seedlings grown on ½ MS plates. The seedlings were ground in liquid nitrogen, and the resulting powder was dissolved in 50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 with protease and phosphatase inhibitor (Thermo Scientific, Rockford, IL, USA). The samples were quantitated using Bradford assay, and equal amounts of lysates were resolved by SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and then incubated with anti-FLAG antibody (Sigma-Aldrich, Saint Louis, MO, USA) or anti-MAPK and anti-phospho-MAPK antibodies (Cell Signaling Technology, Beverly, MA, USA). Horseradish peroxidase conjugated anti-mouse or anti-rabbit secondary antibody were used, respectively, and the immunoblot bands were detected by ECL (Thermo Scientific, Rockford, IL, USA).

**Immunoprecipitation and in-Gel Kinase Assay**

For each immunoprecipitation, 1 mg of extracted proteins (methods in 2.3) were incubated with 30 µL of anti-FLAG M2 resin (Sigma, St. Louis, MO, USA) for 2 h at 4 °C.
on an end-over-end rotator. The mixture was centrifuged at 5,000 g for 30 s, and the supernatant was removed using a narrow-ended pipette tip. The pelleted agarose was then washed three times with 50 mM Tris HCl, 150 mM NaCl, and pH 7.4 for 5 min / time at 4 °C on the rotating system. Following the final wash, 50 µL of 150 ng/µl of 3 X FLAG peptide (Sigma, St. Louis, MO, USA) was incubated with the resin for 30 min at 4 °C. The samples were then centrifuged at 8,000 g for 1 min and the supernatant was recovered without disturbing the pellet. The elution was repeated for another time.

The in-gel kinase assay was carried out according to a previously described method with minor modifications (Zhang et al, 1997). Briefly, the eluted samples were mixed with 2 × Laemmli buffer (Hercules, CA, USA) and then loaded on a 12% polyacrylamide gel with 0.5 mg/mL MKS1 embedded as a substrate. After electrophoresis, the gel was washed three times with the SDS removal buffer (25 mM Tris, pH 7.5, 0.1% Triton X-100, 0.1 mM Na₃VO₄, 5 mM NaF, 0.5 mM DTT and 0.5 mg/mL BSA) at room temperature for 1 h / time. The proteins on the gel were then refolded overnight at 4°C in the re-nature buffer (25 mM Tris-HCl, pH 7.5, 0.1 mM Na₃VO₄, 5 mM NaF, and 1 mM DTT). The gel was then incubated in the kinase reaction buffer (25 mM Tris-HCl, pH 7.5, 10 mM MnCl₂, 1 mM DTT, 0.1 mM Na₃VO₄, 50 µCi γ-³²P ATP (PerkinElmer, Boston, MA, USA), and 200 nM ATP ) at room temperature for 1 h to allow the transfer of ATP to the substrate. The radioactive gel was washed five times with 5% trichloroacetic acid and 1% sodium pyrophosphate for 1 h / time, and then dried using the DryEase® Mini-Gel Drying System (Thermo Scientific, Carlsbad, CA). Subsequently, autoradiograph was obtained by exposing the dried gel to an X-film in a -80 ° freezer.
Immunoprecipitation Coupled with Liquid Chromatography-Tandem Mass Spectrometry (IP-LC-MS/MS)

The IP was performed as described above, except that 10 mg proteins from the wild type Col-0 plants and transgenic plants were used as input. The reagents described in the protocol were scaled up accordingly. After elution, the IP products were fractionated by SDS-PAGE using 12% precast gels (Biorad, Hercules, CA, USA). Gel bands were cut and then washed with 50% acetonitrile in 50 mM ammonium bicarbonate (ABC). Proteins in the gel were first reduced by 10 mM DTT in 100 mM ABC at 37°C for 30 min, and then alkylated by 55mM iodoacetamide in 100 mM ABC at room temperature for 30 min in the dark. The gel pieces were washed first by 50% acetonitrile in 10 mM ABC, and then by 100% acetonitrile and dried by speed vac. The in-gel digestion was performed by adding 5 µg of trypsin (Promega, Madison, WI, USA) in 50 mM ABC, followed by incubation at 37°C for 14 h. The reaction was stopped by adding 1% formic acid / 2% acetonitrile (v/v = 1:1), and the resulting peptides were transferred into a new tube. The digested gel pieces were treated with 60% acetonitrile for another extraction, and all the recovered peptides were dried using speed vac. Peptide samples were cleaned up by solid phase extraction using C18 columns (The Nest Group, Southborough, MA, USA). The desalted samples were enriched for phosphopeptides using Toptip TiO₂ + ZrO₂ (Glygen, Columbia, MD, USA) according to the manufacturer’s instructions.

LC-MS/MS was carried out on an Easy-nLC 1000 system (Thermo Fisher Scientific Inc., Germering, DE, USA) coupled with an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA). The peptides were separated by an Acclaim® PepMap100 C18 column (250 mm × 75 µm; 2 µm-C18) with a linear gradient
of buffer B (0.1 % formic acid, 99.9% ACN) from 2% to 30% for 1h at 350 nL/min. The eluted peptides were ionized by a nano-electrospray ion source. The MS1 scans were performed from 400–2000 m/z at a resolution of 120,000, and the top ten most intense ions were selected for MS2 scan using collision-induced dissociation (CID) at a normalized collision energy (NCE) of 35. A minimum signal threshold of 10,000 and an isolation width at 2 Da were applied to MS2 scans. The charge state dependent electron transfer dissociation (ETD) decision tree was enabled for fragmentation.

**MS Data Analysis**

The raw MS files were processed with Proteome Discoverer version 1.4.1.14 (Thermo Fisher Scientific Inc., Bremen, Germany) using the SEQUEST algorithm. Spectra were searched against the TAIR10 protein database plus the BnMPK4 protein sequence with the following parameters: 10 ppm as mass tolerance for MS1 and 0.02 as mass tolerance for MS2, two as the maximum missed tryptic cleavage sites, carbamidomethylation (+57.021) on cysteine residues as the fixed modification, oxidation of methionine (+15.996) and phosphorylation on tyrosine, serine and threonine (+79.966) as dynamic modifications. Phosphorylation site location was performed by PhosphoRS 3.0 node and then manually checked with Qual browser in Xcalibur (version 2.1, Thermo Scientific) using the raw files. The searched results were further filtered at 1% false discovery rate (FDR) at the peptide confidence level and 2 peptides per protein at the protein identification level.

**Other Methods**

For gene expression analysis, total RNA was extracted from two-week old seedlings using RNAeasy Mini kit (Qiagen, Germantown, MD, USA). The samples were cleaned with the DNA-free DNase treatment & removal reagents (Ambion, Carlsbad,
CA, USA), and the purified RNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilminton, DE, USA). One microgram RNA was used to synthesize the first-strand cDNA using the GoScript reverse transcription system (Promega, Madison, WI, USA). Primers for PCR can be found in Table 3-1.

For root growth measurement, plants were grown on vertical plates. Root length was measured in Image J after taking a picture of the plants along with a meter ruler for calculation.

Results

Overexpression of MPK4 Enhances Resistance to Pst DC3000

We previously infiltrated MPK4 constructs in Nicotiana benthamiana leaves and showed that MPK4 positively regulates ROS production (Zhang et al., 2015b), a hallmark in PTI. To further reveal the physiological relevance of MPK4, transgenic Arabidopsis plants expressing a FLAG-tagged MPK4 were created. Nine independent lines were obtained and confirmed by Western blot. Two lines with similar protein expression levels were selected for further analysis (Figure 3-1A).

In order to determine the immune response in the transgenic plants, forming of bacterial colonies was used to measure the susceptibility to infection of syringe-infiltrated Pst DC3000. While the bacteria growth reached 5.1 cfu/cm² in the wild type Col-0 plants, the accumulation of the virulent pathogen was significantly lower in both transgenic MPK4 line 1 and line 2 (Figure 3-1B). This indicates that overexpression of MPK4 enhances the resistance to Pst DC3000 infection. Mild chlorosis was developed in leaves of the more resistant MPK4 transgenic lines, but not of the Col-0 control (Figure 3-1C), which corroborates the previous observation that the extent of chlorosis could be negatively associated with pathogen resistance (Xu et al., 2006).
In addition to reprogramming plant immune responses, the loss-of-function *mpk4* Arabidopsis mutants also show severe dwarfism and retarded root development (Kosetsu et al., 2010; Petersen et al., 2000). To test whether overexpression of MPK4 in Arabidopsis exerts the opposite effect, we determined the primary root growth of seedlings grown on vertically oriented plates. We found that the root length was comparable in the Col-0 wild type and MPK4 transgenic plants (Figure 3-2), suggesting that a higher level of MPK4 does not necessarily result in apparent changes in root development.

**MPK4 Transgenic Plants Show Disturbed Hormone Regulations**

Previous studies have indicated that changes in susceptibility to *Pst DC3000* of Arabidopsis are primed through JA and SA pathways (Song et al., 2015; Scala et al., 2013). We therefore tested the levels of JA and SA using a targeted metabolomics approach. As shown in Figure 3-3, the level of JA dropped significantly while the SA level was similar in the transgenic plants in comparison to the wild type plants. These data support the idea that the balance between JA and SA in the plant host strongly affects the establishment of bacterial pathogen (Scala et al., 2013). It is also worth mentioning that both the free and their conjugated forms of JA display similar changes. We also extended our measurements of metabolites to other major plant hormones that are involved in plant defense and development (Figure 3-4). We found that epibrassinolide and indole-3-acetic acid are present at a higher and lower level in the transgenic plants, respectively, while abscisic acid, gibberellin and zeatin are present at similar levels to those occurring in the wild type. Thus, overexpression of MPK4 causes up or down regulation of some, but not all phytohormones. These data also suggested
that altered resistance to *Pst* DC3000 in the transgenic plant may be largely due to the suppression of JA signaling.

Up-regulation of pathogenesis related (PR) gene expression is associated with enhanced pathogen resistance (Song et al., 2015). Thus, we also compared the expression levels of PR1 and PR2 in the transgenic lines to those in the wild type plants. The results demonstrated a similar level of expression (Figure 3-5), suggesting that overexpressing MPK4 does not alter the PR gene expression and that the enhanced disease resistance in MPK4 transgenic plants may be independent of PR gene expression. The WRKY40 transcription factor was previously shown to be a negative regulator in resistance to *Pst* DC3000 (Xu et al., 2006). We thus also tested the transcript level of WRKY 40. Similar to the PR gene expression patterns, no significant difference was observed between the Col-0 and the MPK4 transgenic plants.

**MPK4 Transgenic Lines Are More Sensitive In flg22-induced ROS production**

Plant immunity is not only mediated by SA, but is also associated with the production of ROS. Thus, we postulated that the enhanced pathogen resistance in the transgenic plants is correlated with ROS homeostasis regulation. To test this hypothesis, we measured the ROS accumulation in guard cells, since the stomatal pores are the major sites of pathogen entry into plants and MPK4 is preferentially expressed in the guard cells (Berriri et al., 2012; Petersen et al., 2000). While flg22 was able to induce an ROS burst in both Col-0 and transgenic plants, the transgenic lines exhibited an increase in amplitude (Figure 3-6A), indicating that overexpression of MPK4 promotes ROS production in PAMP-induced PTI responses. To discern whether MPK4 functions only in the PTI pathways or if it is also involved in modulating the stomatal aperture, we measured the stomatal size after flg22 treatment. As shown in
Figure 3-6B, the stomatal pores shrank in size significantly in both Col-0 and MPK4 transgenic plants. However, the transgenic plants showed greater reduction in the stomatal pore size. These data suggest that overexpression of MPK4 affects plant immunity by promoting higher levels of ROS production and greater decreases in stomatal size.

**MPK4 is Activated by flg22**

The inducibility of MPK4 activity by flg22 in the wild type Arabidopsis plants prompted us to test whether the heterogeneously expressed MPK4 in the transgenic plants is also activated by flg22. The FLAG-tagged MPK4s were immunoprecipitated from transgenic seedlings treated with flg22. Two approaches demonstrated that MPK4 was activated upon treatment. First, in-gel kinase assay using *B. napus* MPK4 substrate 1 (MKS1) showed that MPK4 is activated at 1 min and peaks in activity at 10 min (Figure 3-7). Second, immunoblot with phos-p44/p42 (an antibody which specifically recognizes the phosphorylated forms of MPK4, MPK3 and MPK6) exhibited a similar activation pattern (Figure 3-7). Notably, the transgenic plants did not demonstrate detectable kinase activity under normal growth conditions (0 min treatment in Figure 3-7).

**MPK4 Phosphorylation Dynamics**

To investigate the phosphorylation status of the inactive epitope-tagged MPK4 at steady state, we employed a LC-MS/MS approach. The FLAG-tagged MPK4 from transgenic plants was immunoprecipitated using anti-FLAG resin and then separated by SDS-PAGE. The corresponding protein band was trypsin digested, and the phosphopeptides were enriched to enhance the possibility of LC-MS/MS identification. The analysis revealed phosphorylation of the tyrosine within the conserved TXY (X
means glutamate in the A, B and C group of MAPK kinases, and aspartate in the D group) activation loop in the peptide N-SETDFMTEYVVTR-C (Figure 3-8A). These in vivo phosphorylation data are in line with the previous finding that recombinant MPK4 expressed in E.coli autophosphorylates on the tyrosine of the activation loop (Zhang et al., 2015b) and that phosphorylation on Tyr is probably due to autophosphorylation (Huang et al, 2000).

Previous studies demonstrated that dual phosphorylation of both the threonine and tyrosine in the TEY motif is required for fully active MAPKs (Ferrell and Bhatt, 1997). We thus determined the phosphorylation status of MPK4 upon flg22 treatment. After 10 min of flg22 elicitation, phosphorylation on the threonine was also observed in addition to the phosphorylation on the tyrosine residue (Figure 3-8). These results support a mechanism for MAPK activation in which phosphorylation on the tyrosine, possibly by autophosphorylation, precedes phosphorylation on the threonine residue.

Previous reports showed that MPKs can be phosphorylated at sites outside the TEY activation loop (Xie et al., 2014). Our analysis identified phosphorylation of MPK4 at Ser195 in control plants (Figure 3-9), a conserved amino acid residue within the VIII domain of MPK4 from different species (Zhang et al., 2015b). Importantly, phosphorylation at Ser195 was not detected in MPK4 transgenic plants under flg22 treatment, providing biochemical data that MPK4 phosphorylation at specific sites is under dynamic changes to regulate the kinase activity.

**Putative Interacting Proteins of MPK4**

To understand how MPK4 modulates various biological processes, we took a proteomics approach to globally identify the putative MPK4 interacting proteins (Figure 3-10). Immunoprecipitation samples from wild type (Col-0) and transgenic plants
carrying MPK4 were analyzed by MS and compared. The Col-0 plants were chosen as a control because the MPK4 transgenic plants were created in Col-0 background. To increase the reliability, proteins identified in at least three out of four replicates in the transgenic plants, but not in the Col-0 control, were considered as putative MPK4 interacting candidates (Table 3-2). Many stress responsive proteins such as heat shock related proteins (HSPs) were enriched in the MPK4-copurifying samples. Other co-immunoprecipitated proteins were key enzymes in photosynthesis and energy metabolic pathways, suggesting a role of MPK4 in controlling plant growth and development. Another very interesting MPK4-interacting candidate was glutaredoxin family protein, an important component of the thiol–disulfide oxidoreductase systems in maintaining intracellular redox state (Meyer et al., 2008). We also identified proteins in cell signaling such as villin 3 as potential MPK4 targets. However, the validity of MPK4-villin 3 interaction, and the physiological relevance remains unknown. Notably, many chloroplast proteins were also co-purified with MPK4. Since MPK4 is localized in the cytosol and nucleus, it is likely that disruption of the organelle boundary during protein extraction process resulted in artificial reaction between MPK4 and some other proteins. Thus, proteins that are not co-localized with MPK4 were labeled with a star in Table 3-2 to reflect a possible unnatural reaction. The localization of proteins in Table 3-2 was obtained from GO Cellular Component from TAIR (https://www.arabidopsis.org/).

Since MPK4 is preferentially expressed in guard cells and it is involved in stomatal movement, we tested the expression pattern of selected MPK4 candidates in B. napus guard cells. The transcript analysis showed that most of the selected genes
have a moderate to strong expression level (Figure 3-11), providing evidence that those proteins could interact with MPK4 in guard cells.

Discussion

Role of MPK4 in Plant Immunity

Arabidopsis MPK4 was proposed to play a negative role in plant defense, since the mpk4 knockout mutant showed enhanced resistance to virulent pathogens including *Pst* DC3000 and *Peronospora parasitica* (Petersen et al., 2000). In contrast to such a role, our analysis of the transgenic Arabidopsis overexpressing BnMPK4 indicates that MPK4 functions positively in suppression of JA signaling, activation of ROS, and enhancement of pathogen resistance. Several possible explanations may account for the discrepancy. First, MPK4 could function in a species-specific manner (Hettenhausen et al., 2012) (Zhang et al., 2015a). For example, silencing of *MPK4* in *Nicotiana attenuata* rendered the plants compromised to ABA-induced stomatal closure (Hettenhausen et al., 2012), while silencing the *MPK4* ortholog in *N. tabacum* led to no apparent changes in ABA-mediated stomatal movement (Gomi et al., 2005).

Furthermore, *MPK4* was cloned from *Brassica napus* Zhongshuang9, a cultivar showing resistant to *Sclerotinia sclerotiorum*. Overexpression of this MPK4 allele in the susceptible cultivar *B. napus* 84039 enhanced plant immunity significantly (Wang et al., 2009). Similarly, alleles of other *MPKs* may exert different functions, as demonstrated by the functional divergence of MPK12 in water use efficiency in various Arabidopsis accessions (Des Marais et al., 2014). Second, the overall plant immunity responses are controlled by an array of both positive and negative players in the balancing of JA and SA signaling, ROS production and PR-gene expression. In the case of MPKs, MPK3 and MPK6 (positive players) and MPK4 (negative player) are activated upon pathogen
infection simultaneously, where a fine-tuning of the antagonizing signals is needed for proper cellular responses (Droillard et al., 2004; Ichimura et al., 2006; Li et al., 2015). Thus, overexpressing a particular MPK may result in unbalanced signaling in plant immunity. Third, the inconsistency between studies using knockout mutants and those employing overexpression lines may stem from the fact that both methods could introduce unexpected pleiotropic effects. This has been observed in studies determining the role of MEKK1 in plant immunity, which has been described as a positive regulator using overexpressing MEKK1 (Asai et al., 2002) and a negative regulator by analysis of the mekk1 mutant (Ichimura et al., 2006). Alternatively, the use of "positive" or "negative" regulator in describing the role of MPKs in plant immunity is misleading because it is only reveals one aspect of the complicated signaling. The discovery of the R protein SUMM2 as a guard protein of MPK4 provides a model that clarifies the role of MPK4 in plant immunity as a positive regulator in PTI (Zhang et al., 2012). In this model, MPK4 is activated in response to pathogen attack to prime PTI. To suppress PTI, pathogens secrete effectors such as HopA1 to reduce MPK4 activity. The disruption of MPK4 activity can be sensed by the guard protein SUMM2, which triggers ETI. Thus, the constitutively activated ETI and higher resistance to Pst DC3000 in the Arabidopsis mutant are results of lacking activity of MPK4, which initiated defense response through SUMM2.

The enhanced resistance to Pst DC3000 in the mpk4 knockout Arabidopsis was suggested as a consequence of an elevated SA level and impaired JA signaling. However, our data indicates that the increased resistance in the transgenic plants is largely due to suppression of JA defense pathway. We speculate that loss-of-function of
MPK4 in Arabidopsis disrupts the balance of JA and SA pathways simultaneously, while overexpression of \( BnMPK4 \) in Arabidopsis majorly targets the JA pathway through an unknown mechanism. Interestingly, Hettenhausen \textit{et al.} showed that SA is not required in immune response against \textit{Pst DC3000} infection in both the wild type and the MPK4 silenced \textit{N. attenuata} plants (Hettenhausen et al., 2012). Moreover, our data indicates that overexpression of MPK4 leads to an enhanced ROS production and a greater reduction in the size of stomatal pores, which is correlated with the increased resistance in the transgenic plants. This supports the idea that MPK4 is a positive, rather than a negative regulator in PTI.

\textbf{Activation of MPK4}

Our data and data from others demonstrated that MPK4, along with MPK3 and MPK6, is activated \textit{in vivo} by flg22 (Droillard et al., 2004; Ichimura et al., 2006). MPK4 can also be activated by osmotic stress. For example, MPK4 is activated at two minutes after the Arabidopsis suspension cells pre-equilibrated in a culture medium of 200 mOsm were transferred to a medium of 15 mOsm (Droillard et al., 2004). Such a hypo-osmolality-triggered MPK4 activation has also been observed in Arabidopsis seedlings (Droillard et al., 2004). In addition, mechanical stresses such as transferring of cells (Droillard et al., 2004) and wounding of the leaves (Gomi et al., 2005) also activates MPK4, presumably through oxidative burst. Moreover, activity of tobacco MPK4 peaked at 10 min after the plants were exposed to 0.2 ppm ozone (Gomi et al., 2005). However, the activation mechanism(s) of MPK4 has been rarely biochemically analyzed \textit{in planta}. We took advantage of the FLAG-tagged MPK4 transgenic plants and showed that both threonine and tyrosine residues in the TEY activation loop are phosphorylated in flg22-triggered activation of MPK4. Importantly, the phosphorylation
of the tyrosine residue at steady state may account for the basal MPK4 activity. Consistent with this, the upstream kinase in the proposed MEKK1-MKK1/MKK2-MPK4 cascade may not have dual-phosphorylation activity, thereby requiring autophosphorylation of MPK4 for activation (Huang et al., 2000). This could also explain why MPK4 can be fully activated quickly at 10 min after challenged with flg22. However, MPK activity is under tight control in plants. Consistent with this idea, the experimentally identified phosphorylation on Ser195 could function as a negative regulation site of the kinase activity, since phosphorylation of this residue was not detected in flg22-treated plants.

It is noteworthy that kinases other than MKK1/2 could be responsible for MPK4 activation, because a growing body of evidence showed that MPK4 functions are not restricted to the single MEKK1-MKK1/2-MPK4 pathway. Similar phenotypes should be expected between mpk4 and mekk1 mutants if the linear cascade is the only signaling pathway of MPK4. Significant differences in salt tolerance and lateral root density, however, were observed in mpk4 and mekk1 (Su et al., 2007). In addition, only mekk1, but not mpk4, displayed a lethal phenotype under standard Arabidopsis growth conditions (Ichimura et al., 2006; Petersen et al., 2000). Thus, it seems more likely that MPK4 and MEKK1 form a kinase cascade that plays an essential role in PAMP response, and MPK4 are involved in other parallel pathways to control processes such as salt stress and root development. This concept is further supported by the finding that an MKK is not the sole kinase that phosphorylates and activates a MAPK in plants. For example, the calcium-dependent protein kinase 18 (CDPK18) phosphorylates and thus activates MPK5 outside of the TXY motif, which was previously thought to be the
activation site of MAPK by an upstream MKK (Xie et al., 2014). Interestingly, phosphorylation sites of MPK5 by CDPK18 are highly conserved between the group A (including MPK5) and the group B MAPKs (including MPK4). Activity of MPK4 was detected in mekk1 mutant (Ichimura et al., 2006), also indicating that other kinases are responsible for the activation. It remains to be seen whether different upstream kinases activate MPK4 through the same phosphorylation sites.

In both yeasts and plants, kinase activity may not be required for MAPK signaling. For example, repression of downstream transcription factors by the yeast MAPK KSS1 is not dependent on the catalytic activity of KSS1 (Bardwell et al., 1998). Similarly, a kinase inactive MEKK1 rescues the dwarf and impaired lateral root development phenotypes in mekk1 (Su et al., 2007; Suarez-Rodriguez et al., 2007).

**MPK4 Interacts with an Array of Putative Targets**

Our data implies that MPK4 interacts with HSP70, consistent with data of others showing a connection between MPK4 and HSPs. For example, both HSP70 and HSP90 showed more than three-fold increase in mRNA in mpk4 compared to the expression levels in wild type Arabidopsis (Petersen et al., 2000). Another pioneering work showed that HSP90 promotes the activation of MPK4 by AvrB, an effector protein secreted by *P. syringae* to manipulate JA levels to enhance plant susceptibility (Cui et al., 2010). This also supports the idea that the HSPs act as molecular chaperones to facilitate folding and interacting of other proteins. Furthermore, the mpk4 dwarf phenotype can be rescued by high temperature, indicating a possible temperature response downstream of MPK4 (Ichimura et al., 2006; Su et al., 2007). High temperature had been demonstrated to suppress the constitutive immune response in the mutants showing HR-mimic phenotypes (Ichimura et al., 2006). It has been shown that R protein
mediated defense is temperature sensitive and that HSP90 interact with RAR1 to regulate the stability of R proteins (Bieri et al., 2004; Hubert et al., 2003). Thus, it is plausible that MPK4 indirectly modulate HR through binding to HSPs and R proteins.

The IP-MS analysis also revealed that MPK4 is associated with proteins in energy metabolism and photosynthesis. However, it is unclear what role MPK4 plays in allocating resources between growth and defense. Retarded growth, extreme dwarfism and constitutively active immune response were observed in Arabidopsis mpk4 mutant (Petersen et al., 2000). In contrast, silencing MPK4 in N. attenuate resulted in significantly increased photosynthesis rate and seed production, as well as enhanced resistance against surface-deposited Pst DC3000 (Hettenhausen et al., 2012). A recent study demonstrated that the chloroplast structure is changed in Arabidopsis mpk4 mutant and that MPK4 positively regulate photosynthesis (Gawronski et al., 2014). Evidence showing the direct association between MPK4 and proteins involved in photosynthesis is lacking. The localization of MPK4 in the nucleus and the cytosol makes the direct interaction between MPK4 and photosynthetic proteins in the chloroplast unlikely. Thus, it is possible that MPK4 modulate the activity of these proteins through other mediators.

We also identified MPK4 as a putative MPK4-interacting protein. Since AtMPK4 and AtMPK11 share 88.3% similarity at the amino acid level, we took extra caution to examine the peptide sequences for MPK11 identification. Indeed, four unique peptides of MPK11 were found to support the presence of MPK11. Both similarity and divergence in function between MPK4 and MPK 11 had been shown in Arabidopsis. For example, MPK11, together with MPK4, has been demonstrated to be involved in governing
cytokinesis (Kosetsu et al., 2010). In addition, MPK11 has been identified as the fourth MPK (the first three being MPK3, MPK6 and MPK4) that is activated by flg22 (Bethke et al., 2012). In contrast to the dwarf phenotype of *mpk4*, the Arabidopsis *mpk11* knockout mutant does not display apparent morphological abnormality (Kosetsu et al., 2010; Hettenhausen et al., 2012). Our data indicated an interaction between MPK4 and MPK11, supporting the idea that MPK4 modulates other MPKs such as MEKK2 (Kong et al., 2012; Su et al., 2013).

Several proteins, not included in our list, have been previously shown to interact with MPK4 (Andreasson et al., 2005; Li et al., 2015; Roux et al., 2015). It is likely that our IP-MS screening system captured only a subset of MPK4 substrates. One explanation is that many kinase-substrate interactions are dependent on upstream stimuli. For instance, interaction and phosphorylation of MPK4 toward ASR3 (Arabidopsis SH4-related 3) occurs when the plants are treated with flg22 (Li et al., 2015). Therefore, it is possible to identify unique substrates under a specific condition. Secondly, although IP-MS/MS provides a robust method for characterizing protein-protein interaction, there remains substantial room for improvement. For example, techniques that can stabilize interacting protein complex should be developed in order to capture weak and transient interactions between signaling molecules such as protein kinases and substrates. Improvements in LC-MS/MS to identify low abundance proteins will also make it more likely to detect proteins such as transcription factors. The workflow used in this study could also identify false positive interacting proteins with MPK4. One source of the false discovery stems from the disruption of organelles during protein extraction, creating an environmental where proteins can interact with another
protein that they never co-localize *in vivo*. Protein extract from specific cellular compartments can be used to avoid such false interactions.
Table 3-1. Primers for transcript analysis

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtPR1_Foward</td>
<td>CTATACACTCTGTGGTG</td>
</tr>
<tr>
<td>AtPR1_Reverse</td>
<td>ATGCCATGTTCCGAC</td>
</tr>
<tr>
<td>AtPR2_Foward</td>
<td>GTGCTCGAGATCGAGGT</td>
</tr>
<tr>
<td>AtPR2_Reverse</td>
<td>GTACCAGATCTGACACAT</td>
</tr>
<tr>
<td>AtWRKY40_Foward</td>
<td>GATCCACGACAAAGTGG</td>
</tr>
<tr>
<td>AtWRKY40.Reverse</td>
<td>AGGGCGTATTGATTCCT</td>
</tr>
<tr>
<td>AtActin7_Foward</td>
<td>GAATGGAATGGAAGCT</td>
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<tr>
<td>AtActin7.Reverse</td>
<td>CTTCTACATGACGATAC</td>
</tr>
<tr>
<td>BnADK1_Foward</td>
<td>CTCGGATCCCGAGGCT</td>
</tr>
<tr>
<td>BnADK1_Reverse</td>
<td>CCTCGAGGGGGTGAAGT</td>
</tr>
<tr>
<td>BnCPN60A_Foward</td>
<td>CCAAGCTTTGCCAGATG</td>
</tr>
<tr>
<td>BnCPN60A.Reverse</td>
<td>CCAATCTCGCTTGGAT</td>
</tr>
<tr>
<td>BnRPS5_Foward</td>
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</tr>
<tr>
<td>BnRPS5_Reverse</td>
<td>CCAATCTCGCTTGGAG</td>
</tr>
<tr>
<td>BnVIN3_Foward</td>
<td>CTCGGATCCCGAGGAG</td>
</tr>
<tr>
<td>BnVIN3.Reverse</td>
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<tr>
<td>BnGTR_Foward</td>
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<td>BnGTR.Reverse</td>
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<td>BnRBOHD_Foward</td>
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<td>BnRBOHD.Reverse</td>
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<td>BnRBOHF_Foward</td>
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<td>BnRBOHF.Reverse</td>
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</tr>
<tr>
<td>BnMDG_Foward</td>
<td>CTCGGATCCCGAGGAG</td>
</tr>
<tr>
<td>BnMDG.Reverse</td>
<td>CTCGGATCCCGAGGAG</td>
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### Table 3-2. The identified putative MPK4 interacting proteins

<table>
<thead>
<tr>
<th>Accession</th>
<th>Protein</th>
<th>Unique peptides</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT2G28000</td>
<td>Chaperonin-60 alpha</td>
<td>ELFETDSVYDSEK; APAAAAPEGLMV; LSGGVAVIK; NVVLDEFGSPK; LGADIIVQK; VVNDGVTIAR; VGAATETELEDR; TNDSAADGTTTASILAR</td>
<td>Stress responsive</td>
</tr>
<tr>
<td>AT1G56070</td>
<td>Elongation factor G</td>
<td>NATLTNEKEVDAHP; RVIYASQITAKPR; GGGQAPIVTAR; FSVSPVVR; EGPLAEENMR;</td>
<td>Translation factor activity</td>
</tr>
<tr>
<td>AT5G02500</td>
<td>Heat shock cognate protein 70</td>
<td>ATAGDTHLGEDFNR; TTPSYAFTDSER; NOVAMNPVNTVFDAK; NALENYAYNMR; DAGVIAGLNVmR</td>
<td>Stress responsive</td>
</tr>
<tr>
<td>AT1G76030*</td>
<td>ATPase, V1 complex, subunit B protein</td>
<td>AVQVVFEGTSGIDNK; TVSGVAGPLVLDK; TPVSLDMGLR; QIYPPINVPSLSR; GQVLEVDEGK; TLDQFYSR; YQEIVNIR</td>
<td>Stress responsive</td>
</tr>
<tr>
<td>AT4G33010*</td>
<td>P-protein 1 of glycerol decarboxylase</td>
<td>GNAVQNNVLK; IIGISVDSGK</td>
<td>Metabolic processes</td>
</tr>
<tr>
<td>AT3G09820</td>
<td>Adenosine kinase 1</td>
<td>SLIANLSAANCYK; AGCYASNVVIOR; LNNAILAEDK; VHGWTDDVEQIAIK</td>
<td>ATP dependent phosphorylation</td>
</tr>
<tr>
<td>AT1G04410</td>
<td>Lactate/ malate dehydrogenase family protein</td>
<td>SLLVANPANTNALILK; NVIWGNHSSQYPDNHAK; EFAPSIPEK; MELDAAFPLLK; NGDWIIVQPLPDEVSR</td>
<td>Metabolic processes</td>
</tr>
<tr>
<td>AT5G09660*</td>
<td>NAD-malate dehydrogenase 2</td>
<td>AGAGSATLSMAYAAK; KLLGVTTLDVAR; LLGVITTDVAR; IQNNGTEVEAK; TGAEEVYQLPnEYER; IQnNGTEVEAK; FADAcLR</td>
<td>Energy metabolism</td>
</tr>
<tr>
<td>AT1G79930</td>
<td>Heat shock protein 70-15</td>
<td>FIGTAGAASTMMPNPK; GCAQLCAILSPTFK; NAVESYVYDMR; FIGTAGAASTMPPNPK; ISTYTIQPFQSSK; YATPALSADVK</td>
<td>Stress responsive</td>
</tr>
<tr>
<td>AT1G09340*</td>
<td>Chloroplast RNA binding</td>
<td>HKGKLETESLLQSK; GKELESSLLQSK; QLPGESDQFADFSSK; EADFTTDDMLSK; EAAEVEPIEALPK; AGGFPEPEIHYNPK; EGHQVTLFTR; ILIMGGR; EIFNISGEEK</td>
<td>Involved in MAPK cascade</td>
</tr>
<tr>
<td>AT3G57410</td>
<td>Villin 3</td>
<td>ITEGNEPCFTTYFSWSTDSTK; ATVQGNSYQKK; AAAVAALSQVLTAEKKK; GSSGETYPEISIALQVSGTVHNNK; AEALALTSANSSSPSSK</td>
<td>Ca²⁺-regulated actin-binding protein</td>
</tr>
<tr>
<td>Accession</td>
<td>Protein</td>
<td>Unique peptides</td>
<td>Annotation</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>AT3G06650</td>
<td>ATP-citrate lyase B-1</td>
<td>VVAIIAEGVPESDTK; FGGAIDDAAR</td>
<td>Carbohydrate metabolism</td>
</tr>
<tr>
<td>AT4G38970*</td>
<td>Fructose-bisphosphate aldolase 2</td>
<td>TWGGRPENVNAAQTTLAR; RLDSIGLENTCEANR; LDSIGLENTCEANR; GILAmDESNAcTcGK; ATPEQVAAYTLK</td>
<td>Metabolic processes</td>
</tr>
<tr>
<td>AT5G40370</td>
<td>Glutaredoxin family protein</td>
<td>LVPLLTEAGAIAGK</td>
<td>Redox homeostasis regulation</td>
</tr>
<tr>
<td>AT4G18480*</td>
<td>P-loop-containing nucleoside triphosphate hydrolases</td>
<td>EKVKEGQVPVIAKT; ANLSSVQIDR; ANLSSVQIDRELK; FGMHAQVGTVR; FGMmHAQVGTVR</td>
<td>ATPase activity</td>
</tr>
<tr>
<td>AT3G56240*</td>
<td>Copper chaperone</td>
<td>VETVTETKTEAETKEAK; AEADPKVETVETKTEAETKEAK; GNVEPEAVFQTSDK; AAEAEATKPSQV; AAEADPKVETVETK</td>
<td>Stress responsive</td>
</tr>
<tr>
<td>AT1G58230*</td>
<td>Beach-domain homolog B</td>
<td>cSYPKVTDLtscLIGtR; LVELSSEDnlFLsHPcR; GSKPLMHPttaERPSR; sELLnhDDDLALNEQHFVR</td>
<td>WD40 and Beach domain-containing protein</td>
</tr>
<tr>
<td>AT1G03130*</td>
<td>Photosystem I subunit D-2</td>
<td>EQIFEMPTGGAAIMR; SIGKVNSPIEVK;</td>
<td>Photosynthesis</td>
</tr>
<tr>
<td>AT5G06290*</td>
<td>2-cysteine peroxiredoxin B</td>
<td>SGGLGDLYPLVSDITK; KSGGLGDLYPLVSDITK; SFGVLIPDQGIALR;</td>
<td>Redox homeostasis regulation</td>
</tr>
<tr>
<td>AT5G23060</td>
<td>Calcium sensing receptor</td>
<td>VFQVVGDKPALDTALPIAK; AQEAmQSSGFDSEPVFNAAK; RVEAEIAALK; VISIPLEELPNVK; LASPAFSEASKK; TVTDVQAQTSK; NoYIVTGDGFSGGR; LASPAFSEASKK; DQIVSSSLTEVEK VISIPLEELPNK</td>
<td>Modulates cytoplasmic Ca(^{2+}) concentration stomatal movement</td>
</tr>
<tr>
<td>AT3G52960</td>
<td>Thioredoxin superfamily protein</td>
<td>VLNLEEGAAFTNSSAEdmLK; YAILADDGVK; LGVELDLR; GIDVIAcISvDAnFAVMEAWR;</td>
<td>Redox homeostasis regulation</td>
</tr>
<tr>
<td>AT1G01560</td>
<td>MAP kinase 11</td>
<td>SNQPLTDDHSR; MLVFDPrR; mLVFPnR; YVRQLPQYPR</td>
<td>Stress signaling</td>
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<tr>
<td>AT5G07200</td>
<td>Gibberellin 20-oxidase 3</td>
<td>MGDyEDFGKVVQYEAEAmnTLSLK; ETLSFKsPEEKIHsQlVK; YPDFTWSM FLEFTQ; VYQEyAEAMNTLSLK</td>
<td>Catalyzes oxidation in GA biosynthesis</td>
</tr>
<tr>
<td>AT2G21660</td>
<td>Cold, circadian rhythm, and RNA binding 2</td>
<td>ALETAFAQYGDVDSK; GFGFVTFKDEK</td>
<td>A glycine-rich RNA binding protein that regulates the circadian clock</td>
</tr>
</tbody>
</table>

Note: In the column of unique peptide sequence, c, m and n denote carbamiodomethylation of cysteine, oxidation of methione and deamindation of asparagine residues, respectively.
Figure 3-1. Analysis of immune response to *Pst* DC 3000 infection in transgenic plants expressing MPK4. A) Identification of MPK4 transgenic lines. Protein extracts from indicated plants were analyzed by immunoblot (IB) using anti-FLAG antibody (upper panel), and Ponceau staining (Ponc) of the Rubisco large unit was used as a loading control (bottom panel). B) Overexpression of MPK4 enhances resistance to *Pst* DC 3000 infection. Four-week old plants were infiltrated with P.s.t DC 3000 at a concentration of OD600 = 0.0001, and bacterial growth assay was performed three days after infiltration on indicated genotypes. Three individual leaves was combined as one replicate and six biological replicates were performed. Error bars represent standard deviation, and * indicates p < 0.05 statistically when comparing to the Col-0 wild type. C) Syringe-infiltrated Arabidopsis leaves. Chlorosis on the transgenic plants were indicated by white arrows.
Figure 3-2. Primary root growth was not altered in MPK4 transgenic plants. A) Ten-day-old seedlings of the Col-0 and MPK4 transgenic Arabidopsis lines. B) Comparison of the primary root length between the MPK4 transgenic lines and the Col-0. Data represented measurement of 40 individual plants in each background, and the standard deviation was shown as the error bars. Student’s t-test showed no statistically difference between the transgenic lines and the Col-0 at 0.05 significance level.
Figure 3-3. Overexpression of MPK4 reprograms hormone regulation. The plant hormone level was measured by multiple reaction monitoring. The relative intensity was expressed in thousands on the y axis. Error bar indicates standard deviation from three independent experiments, and * denote p < 0.05 comparing with the Col-0 using Student’s t test. JA: jasmonic acid; JA-Ile: JA-isoleucine conjugate; MeJA: methyl jasmonate; DHJA: 9,10-dihydrojasmonic acid; MeDJA: 9,10-dihydrojasmonic acid methylester; SA: salicylic acid.
Figure 3-4. Analysis of other major phytohormones in MPK4 transgenic plants. For each of the indicated hormone or their derivatives, the relative levels were presented as intensity (in thousands) using white bar (left) for Col-0, grey bar (middle) for MPK4-line 1, and black bar (right) for MPK4-line 2. ABA: abscisic acid; ABA-GE: abscisic acid-glucose-ester conjugate; BR: epi brassinolide; GA₃: gibberellines A3; GA₄: gibberellines A4; IAA: indole-3-acetic acid; I3CA: indole-3-carboxylic acid; IBA: indole-3-butyric acid; IA: linolenic acid; PA: phaseic acid; TA: traumatic acid; tZTG: trans-zeatin-glucoside conjugate; tZTR: Trans-zeatin riboside conjugate; MeAc: methyl indol-3-acetate.
Figure 3-5. Transcript analysis in Col-0 and MPK4 transgenic lines. Semi-quantitative RT-PCR was performed with mRNA extracted from two-week-old seedlings. Actin was used as a loading control.
Figure 3-6. Stomatal movement and ROS accumulation induced by flg22 in the Col-0 and MPK4 transgenic plants. A) Stomatal closure induced by flg22. The size of stomatal aperture was the average of three biological experiments, each with measurement from at least 30 guard cell pairs. Error bars represent standard error. B) ROS production induced by flg22. Quantification of ROS level in guard cells. The relative intensity represents the average ± standard error of three biological experiments, at least 30 stomata per experiment. Different letters above the bar indicates statistically significant difference at .05 confidence level.
Figure 3-7. MPK4 is activated by flg22. Total proteins were extracted from two-week seedlings treated with 10 μM flg22 for 1, 5, 10 and 30 min. MPK4 was pulled down by anti-FLAG resin. Activation of MPK4 was analyzed by in gel kinase assay with MKS1 as a substrate (top panel) and by immunoblot with anti-phospho-MPKs (middle panel). Immunoblot with MAPK antibody was used as a loading control (bottom panel).
Figure 3-8. Representative MS/MS spectra showing the phosphorylation of MPK4. The phosphorylation events were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The b (in blue) and y (in red) ion series of the fragmented peptides were labeled in the MS/MS spectra to localize the phosphorylation site, and the loss of \( \text{H}_2\text{O} \), \( \text{NH}_3 \), and \( \text{H}_3\text{PO}_4 \) were also denoted. A) Phosphorylation of MPK4 at the tyrosine in the TEY activation loop under steady state. B) Phosphorylation of MPK4 at the tyrosine residue in the TEY motif with another peptide that has a missed cleavage site for trypsin. C) Phosphorylation of MPK4 at the threonine TEY activation loop elicited by flagellin peptide flg22.
Figure 3-9. Phosphorylation of MPK4 outside the TEY activation loop. MS/MS spectra of a peptide that has a phosphorylation site outside of the TEY motif. Labels of the spectra were the same in Figure 5.
Create transgenic Arabidopsis
Overexpressing MPK4 - 3 x FLAG

↓
Protein extraction & Immunoprecipitation

↓
SDS-PAGE

↓
Trypsin digestion

↓
Liquid chromatography with tandem mass spectrometry

↓
Data analysis

Figure 3-10. Schematic diagram of identifying MPK4-interacting proteins. The FLAG-tagged MPK4, along with the interacting proteins, were immunoprecipitated from the MPK4 overexpressing lines. The elution from the immunoprecipitation was fractionated and cleaned-up by SDS-PAGE. The resolved protein bands were cut from the gel and digested by trypsin. The resulting peptide samples were analyzed by liquid chromatography with tandem mass spectrometry.
Figure 3-11. Transcript analysis of putative MPK4-interacting proteins encoding genes in *B. napus* guard cells. RNA was extracted from guard cells and the gene expression was detected by RT-PCR. **GRX**: Glutaredoxin family protein; **MDH**: malate dehydrogenase; **ADK1**: Adenosine kinase 1; **RBOH D**: Respiratory burst oxidase homolog D; **RBOH F**: Respiratory burst oxidase homolog F; **CPN 60 α**: Chaperonin-60 alpha; **RPS5**: Ribosomal protein S5; **VIN3**: Villin 3. Red arrows indicate the expected band of PCR products.
CHAPTER 4
IDENTIFICATION OF THIOREDOXIN TARGETS IN GUARD CELL ENRICHED EPIDERMAL PEELS USING CYSTMT PROTEOMICS

Background

Cysteine modifications mediated by thioredoxin (Trx) represent an important mechanism for rapid control of cellular activities in plants, such as photosynthesis* (Hall et al., 2010), seed germination (Alkhalifioui et al., 2007) and pollination (Yamamoto and Nasrallah, 2013). Different Trx isoforms, f, h, m, o, x and y, in various cellular compartments have been described in plants (Meyer et al., 2008; Montrichard et al., 2009), and there are at least 20 Trx genes in the model plant Arabidopsis thaliana (Meyer et al., 2005). Due to the number of Trx isoforms and diverse interacting partners, the plant Trx systems are complex. For example, reversible reduction of oxidized proteins could be carried out by a specific Trx paired with a specific NADPH-dependent Trx reductase (TrxR) (Montrichard et al., 2009). Alternatively, Trx-mediated reduction can also coupled with ferredoxin–thioredoxin reductase (FTR). The FTR/thioredoxin system is only present in photosynthetic organism that can transfer electrons to ferredoxin using light energy (Montrichard et al., 2009). Interestingly, Trx h can use both TrxR and FTR in an array of physiological contents, highlighting their importance in plants.

Knowledge of Trx target proteins is fundamental for understanding Trx-controlled redox-regulated physiological processes. Two main approaches, one based

* Partial content of this chapter was reprinted with permission from Journal of Proteomics, 2016, 133:48-53.
on the covalent binding between a Trx mutant and target proteins, and the other based on specific thiol labeling, have been developed to identify Trx targets (Buchanan, 2005; Hagglund et al., 2008; Marchand et al., 2004; Marchand et al., 2006; Montrichard et al., 2009; Yamazaki et al., 2004). The first method uses a Trx mutant with the second cysteine of the conserved CxxC active site replaced by serine or alanine to trap potential targets via affinity binding from cell lysates. Protein eluted with strong reducing reagents such as DTT are then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Balmer et al., 2004b; Yamazaki et al., 2004) or by two-dimensional gel electrophoresis (2-DE) (Balmer et al., 2003; Yamazaki et al., 2004), followed by identification using mass spectrometry (MS). The sites of cysteine modifications and the quantitation of the redox status, however, are missing from such an approach. In the second approach, fluorescent thiol group-specific probes, such as monobromobimane (mBBr) (Hall et al., 2010) or cyanine 5 maleimide (Cy5m) (Maeda et al., 2004), are used to label free sulfhydryl groups (-SH). Then the probe-labeled proteins are resolved in 2-DE to reveal potential Trx targets through image analysis. Disadvantages of this method include low sensitivity and low labeling efficiency, as well as the limitations of 2-DE techniques. A non gel-based thiol labeling workflow, i.e., isotope-coded affinity tag (ICAT) has also been applied in Trx targets identification, providing both the sites of the redox-sensitive cysteines and the quantitation of the redox status of individual peptides (Hagglund et al., 2008). However, there are only two tags (i.e., heavy and light), limiting the number of samples that can be analyzed simultaneously. These traditional approaches have been employed to identify Trx
targets in plants, and a total of over 500 potential targets have been discovered in various plant species and tissues (Montrichard et al., 2009).

Although hundreds of Trx targets have been identified using different approaches, the capture of targets in a quantitative and efficient manner is challenging. In this study, a high-throughput method using cysteine reactive tandem mass tag (cysTMT) labeling followed by liquid chromatography (LC)-mass spectrometry (MS) to screen for Trx targets was developed. Compared to existing methods, this approach allows for i) three replicates of pairwise comparison in a single LC-MS run to reduce run-to-run variation; ii) efficient enrichment of cysteine-containing peptides that requires low protein input; iii) accurate quantification of the cysteine redox status and localization of the Trx targeted cysteine residues. Application of this method in guard cell-enriched epidermal peels from *Brassica napus* revealed 80 putative Trx h targets involved in a broad range of processes, including photosynthesis, stress response, metabolism and cell signaling.

**Materials and Methods**

**Plant Growth, Epidermal Peels Preparation, and Protein Extraction**

*Brassica napus* plants were grown under conditions as previously described (Zhang et al., 2015; Zhu et al., 2009). To collect guard cell-enriched epidermal peels, fully expanded leaves from 7-8 weeks old plants were excised with main vein removed and then blended for 1 min in cold tap water with intermittent stops. The blended mixture was then filtered through a mesh with 100 µm pores to remove the cell debris. Epidermal peels were then washed thoroughly with deionized water and dried briefly with filter paper. The purity of intact stomatal guard cells was over 95% with
contaminating pavement cells. The peels were frozen in liquid nitrogen immediately and stored in -80 °C for future use.

The collected samples were ground into fine powder with liquid nitrogen, and proteins were extracted with 50 mM Tris-HCl, 1 mM EDTA, 0.1% Triton X-100 and protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA), pH 7.4. The samples were centrifuged at 20,000 g for 30 min at 4 °C, and the supernatants were transferred and quantified using Bradford protein assay (Bio-Rad Laboratories, Los Angeles, CA, USA). The protein crude extract was rotated on an end-over-end shaker overnight at 4 °C to allow sufficient oxidization of the proteins by air (Hagglund et al., 2010). To quantify the efficiency of air oxidation, protein samples before and after overnight treatment were assayed with Ellman’s Reagent (5,5-dithio-bis-(2-nitrobenzoic acid, DTNB). Briefly, 100 µg of the samples were incubated with 0.1 mM DTNB in 100 mM Tris-HCl, pH 7.5 for 2 min at room temperature. The contents of free sulfhydryl were measured at 412 nm using a spectrophotometer. The pre-oxidized samples were also blocked with 100 mM iodoacetamide and were assayed again with DTNB to ensure complete oxidization of free sulphydryl groups.

**Thioredoxin Reaction, CysTMT Labeling, and LC-MS/MS**

*B. napus* thioredoxin h isoform (Trx h) was cloned and expressed in *E. coli*. Activated Trx h was obtained by incubation with 1 mM DTT, followed by purification using a nickel column to capture His-tagged Trx h (Affymetrix Inc., CA, USA) (Zhang et al., 2015). The activity of purified Trx h was tested by incubating with pre-oxidized epidermal protein extract, and the newly formed free sulphydryl groups were measured using DTNB.
For Trx h target identification, total protein samples from cell lysates were oxidized by air to induce disulfide bond formation (Hagglund et al., 2010). The oxidized protein sample was aliquoted into six tubes, each with 100 µg protein. Three of the aliquots were incubated with 25 µg Trx h for 1 h at room temperature and the other three with 25 mM Tris-HCl, pH 7.4 as controls. After the redox reaction, iodoacetamide (IAM) was added into each reaction mixture at a final concentration of 100 mM to block free thiol groups. The alkylation step was conducted at 37 °C for 1 h in the dark (Zhu et al., 2014). Acetone was then added to a final concentration of 80% (v/v) to precipitate the proteins and to remove extra IAM and the mixture was incubated for overnight at -20 °C. The samples were centrifuged at 20,000 g for 30 min at 4 °C, and the pellets were collected and washed twice with ice-cold 80% acetone. After brief lyophilization to remove acetone, the pellets were dissolved in 6 M urea, 50 mM Tris and 1 mM EDTA. Disulfide bonds in the samples were then reduced by 1 mM Tris (2-carboxyethyl) phosphine (TCEP) at room temperature for 1 h. The reducing reagent was removed using a ZebaTM spin desalting column (Thermo Fisher Scientific Inc., IL, USA). The three control samples were labeled with cysTMT tag 126, 127, and 128, and the Trx treated samples were labeled with 129, 130, and 131, respectively, for 2 h at room temperature. All samples were combined after labeling and resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was cut into different sections and in-gel digestion with trypsin was performed (Chen, 2006). Peptides containing cysTMT were enriched using anti-TMT resin according to the manufacturer’s instructions.
Peptide samples were analyzed on a Q Exactive™ Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA) with a nano-electrospray ion source. Peptides were loaded onto an Acclaim® PepMap™ micro-Precolumn (20 mm × 75 μm; 3 μm-C18) using an automated Easy-nLC 1000 system (Thermo Fisher Scientific Inc., Germering, DE, USA), and then separated with an Acclaim® PepMap100 C18 column (250 mm × 75 μm; 2 μm-C18) with acetonitrile (ACN) gradient up to 25% containing 0.1% formic acid (v/v), for 60 min at 400 nL/min. The LC-coupled MS was operated in data-dependent manner with full MS scan from 400–2000 m/z at resolution of 70,000, and MS/MS scan at resolution of 17,500. The five most intense ions were selected for fragmentation using higher-energy collisional dissociation (HCD) at normalized collision energy (NCE) of 28. The fixed first mass was set as 115 m/z to capture the cleaved cysTMT tags in the low m/z region. MS/MS scan was acquired with minimum signal threshold 1E+5 and isolation width at 2 Da. Maximal filling times were 100 ms for the full MS scans and the MS/MS scans.

**Database Searching and Analysis**

The acquired raw files were processed by Proteome Discoverer version 1.4.1.14 (Thermo Fisher Scientific Inc., Bremen, Germany). The spectra were searched by SEQUEST against NCBI green plant non-redundant database with 890629 entries. The SEQUEST parameters allowed maximum of two missed tryptic cleavage sites, with cysTMT modified cysteine (+304.177 Da) and carbamidomethylation (+57.021) on cysteine residues, as well as oxidation of methionine (+15.996), as dynamic modifications. For confident peptide identification, the false discovery rate (FDR) was set as 1%. CysTMT modified peptides were obtained through filtering from all the identified high confidence peptides. The resulting peptides were then ungrouped, and
the quantification value for each cysTMT tag was then acquired. The raw data were exported, and the redundant peptides were combined using the aggregate function in R (version 3.0.2). The intensity of each channel for the same peptide was normalized against the peak area sum of all peptides in the same channel. The normalized ion intensities of all tags for each peptide were then log$_2$ transformed to perform Student $t$ test to reveal the peptides statistically changed between control and treated groups. Fold change of each cysteine-labeled peptide was calculated as the ratio between the mean of the normalized tag intensities in each group. A peptide was considered to be Trx-reactive with two criteria: 1) fold change larger than 1.22, a critical value widely used in previous Trx work (Montrichard et al., 2009); 2) $p$ value in Student $t$ test smaller than 0.05, the significance level chosen in this study. For protein identification, the following criteria were applied: peptide at 1% FDR, and at least two unique peptides per protein.

**Results**

**Design and Rationale of the Workflow**

To identify Trx targets quantitatively in a more efficient manner, we implemented a new approach to differentially label cysteine residues with cysTMT (Figure 4-1). The cysTMT reagents contain six tandem mass tags that react specifically with sulfhydryl groups and have been used to quantify modification on cysteine residues (Murray et al., 2012). We applied this method using protein extract from *Brassica napus* guard cell-enriched epidermal peels, in which redox regulation plays essential roles in cellular signaling and metabolism (Kollist et al., 2014; Zhang et al., 2014; Zhu et al., 2014; Zhu et al., 2010). Briefly, total protein samples from cell lysates were oxidized by air to induce disulfide bond formation (Hagglund et al., 2010). Activated (reduced) Trx h was
then incubated with the oxidized samples to reduce free thiol groups of target proteins, whereas the introduction of Trx h had no effect on non-target proteins. The newly formed -SH groups were immediately and irreversibly alkylated with iodoacetamide (IAM). After acetone precipitation overnight and brief lyophilization of the protein pellets, the remaining disulfide bonds were then reduced by the strong reducing agent Tris (2-carboxyethyl) phosphine (TCEP), which doesn't interfere with the downstream cysTMT reagents. All the available free thiols were labeled with cysTMT tags: 126, 127 and 128 for the control groups without Trx h treatment, and 129, 130 and 131 for the three replicates with Trx h. All the samples were combined and separated by SDS-PAGE. The gel lane was cut into nine sections of similar size, and the fractionated protein samples were digested with trypsin. The cysTMT labeled peptides were enriched by incubation with an immobilized anti-TMT antibody resin for 2 h at room temperature. Peptides were eluted with 50% acetonitrile and 0.4% trifluoroacetic acid for LC-MS/MS analysis. Peptide samples were analyzed using a Q ExactiveTM Orbitrap mass spectrometer, and the raw data were searched using Proteome Discoverer 1.4.

In this method, the quantitative values, i.e., peak intensities of the cysTMT tags in the low m/z range in MS/MS spectra were used to determine the redox status of cysteines, thus indicating putative Trx targets (Figure 4-1). For the non-target proteins, the same levels of cysTMT labeling with Trx treatment (129, 130, and 131) and without (126, 127, and 128) were expected. Thus, the respective ratios of 129, 130, and 131 to 126, 127, and 128 should theoretically be close to 1. Because cysteines in target proteins were reduced by Trx and then irreversibly blocked by IAM, less labeling of these cysteines compared to those of the control groups occurred. The ratios between
control and Trx-treated samples of the Trx-targeted cysteines, therefore, are greater than one (Figure 4-1). Statistically, a ratio greater than 1.22 was deemed sufficient to define a Trx reduction event in previous experiments (Montrichard et al., 2009). The ratio for Trx itself is expected to be less than one, as a result of the catalytic motif being oxidized.

**Tests of Key Steps in the Workflow**

A gentle *in vitro* oxidization of extracted proteins by air was used in this study. Exposure of the samples to air overnight effectively oxidized the proteins, as the contents of free thiols measured by 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) dropped significantly after the overnight oxidation (Figure 4-2). The measurement of free thiols in the oxidized samples with and without IAM blocking were not statistically different, indicating that air oxidation of free thiols was complete (Figure 4-2).

To confirm the activity of Trx *h*, both oxidized and reduced Trx *h* were incubated with the pre-oxidized samples. The results showed that only the reduced Trx *h* was able to reduce the oxidized thiols, giving rise to free thiols and thus a significant increase in the DTNB measurement (Figure 4-2).

Another key step in the workflow is the alkylation after Trx *h* reduction. Insufficient alkylation of the newly formed sulphydryl groups by Trx *h* would render the cysteine containing peptides of Trx *h* target proteins the same levels of labeling by cystTMT tags, thus resulting in a false negative discovery of some Trx *h* targets. To ensure a high IAM alkylation efficiency, samples after Trx *h* reduction were precipitated with acetone and digested, the resulting peptides were analyzed by LC-MS/MS. At 1% false discovery rate (FDR) level, 118 out of the 122 cysteine containing peptides were
found as modified by carbamylation. Thus, the alkylation efficiency was 96.7% in this workflow.

**Validation of the Workflow Using Enriched Guard Cells**

Known Trx targets only represent a small fraction of the whole plant proteome (Montrichard et al., 2009). Thus, the *in vitro* Trx reaction should not alter the redox status of majority of the proteins, giving rise to an average ratio of all available cysteine residues labeled by cysTMT between the control and the Trx-treated groups to be close to 1. Experimental data from this study showed an average ratio of 1.04, indicating that the labeling strategy introduced no bias and is appropriate for Trx target identification.

The workflow resulted in identification of 767 non-redundant peptides with cysTMT tags at 1% FDR. Compared to the 199 peptides identified from the ICAT labeling experiment (Hagglund et al., 2008), the dramatic increase in identified cysteine-containing peptides may be attributed to the enrichment effect of six-plex cysTMT, which allows for the identification of low-abundance proteins. The number reported here is also consistent with the low occurrence of cysteines in plant proteome. For example, cysteine only represents 2% of the amino acid residues in the proteome of the model plant Arabidopsis (Montrichard et al., 2009).

Using criteria of fold-change of 1.22 as a cutoff and of statistical analysis at the significance level of 0.05, 80 putative Trx *h* targets were identified for the first time in guard cell enriched samples (Table 4-1). The cysteine-containing peptide for one representative substrate is shown in Figure 4-2, with the intensity of cysTMT channels labeled. Interestingly, a broad range of Trx targets were identified here, with ~ 60% (46 out of 80) being previously reported in other cells or tissues, and ~40% (34 out of 80) being newly identified targets (Table 4-1).
The Identified Trx h Targets Are Involved in a Broad Range of Physiological Processes

Functional classification of the 80 putative Trx h target proteins revealed that 31 participate in energy and metabolic processes. Significantly, 18 of those targets have been previously identified (Table 4-1) (Alkhalfioui et al., 2007; Balmer et al., 2004a; Balmer et al., 2006a; Balmer et al., 2004b; Hagglund et al., 2008; Lemaire et al., 2004; Lindahl et al., 2007; Marchand et al., 2006; Schurmann and Buchanan, 2008; Wong et al., 2004). Amino acid metabolism has been demonstrated to be regulated by Trx; in particular, aminotransferases are reduced by Trx (Balmer et al., 2006a; Marchand et al., 2006; Wong et al., 2004). Interestingly, this study identified a γ-glutamylcysteine synthetase, which catalyzes the first step in the biosynthesis of the cellular antioxidant glutathione (GSH). In addition, cysteine synthetase was also reported to be Trx target (Balmer et al., 2004b). This finding of Trx regulation of γ-glutamylcysteine synthetase and thus GSH biosynthesis is new. It will be intriguing to further investigate this new Trx interaction and its physiological significance in maintaining cellular GSH levels.

Another major group of the Trx h targets participates in protein synthesis, folding, transport and degradation. A substantial number of chaperonin, isomerase, and lipid transfer proteins were repetitively identified as Trx targets (Alkhalfioui et al., 2007; Balmer et al., 2006a; Hagglund et al., 2008; Hajheidari et al., 2007; Maeda et al., 2003; Maeda et al., 2004; Wong et al., 2004), highlighting the key role of Trx in protein turnover and transport between cellular compartments. Notably, aspartic protease in guard cell 1 (ASPG1) was identified with a reduced-to-oxidized ratio of 2.4, indicating extensive reduction by Trx in vitro. ASPG1 functions in abscisic acid (ABA) mediated drought response, and overexpression of ASPG1 reduced water loss in Arabidopsis.
(Yao et al., 2012). Reactive oxygen species (ROS) burst and redox regulation of key kinases represent important signaling events in ABA signal transduction (Zhang et al., 2014; Zhu et al., 2014; Zhu et al., 2010). However, the role of Trx in this process remained elusive. The identification of ASPG1 as the potential Trx target may open the door to fill this knowledge gap.

The majority of Trx h targets in stress and defense, signaling, cell cycle, and cell structure are novel Trx substrates. Guard cells are rich in cell wall pectin and polysaccharides that contribute to the stomatal movement (Jones et al., 2003; Merced and Renzaglia, 2014). The cell wall localized pectin acetyylesterase catalyzes the interaction between acetylated polysaccharides and cellulose (Orfila et al., 2012), a key process governing the cell structure. Redox control in plant growth and development has been widely recognized (Kocsy et al., 2014). Yet such regulatory mechanism in cell wall structure, especially in guard cells, has not been reported. Significantly, the cell wall and the surrounding apoplast represent a relative more oxidized environment due to the low antioxidant-buffering capacity, comparing to a more reduced environment in the cytosol (Foyer et al, 2013). Interestingly, it has been demonstrated that Trx h from *Nicotiana alata* can be secreted into the extracellular matrix (Juárez-Díaz et al, 2006) and that Trx h from Arabidopsis moves between different cells in the root (Meng et al, 2010). Currently, it is not clear how an extraplastic-localized Trx interacts with a cell wall protein. However, the possibility of artificial interaction between Trx and pectin acetyylesterase can not be ruled out in this study.

Enzymes controlling redox homeostasis are well-known Trx targets (Schurmann and Buchanan, 2008; Wong et al., 2004; Yamazaki et al., 2004). For
example, 2-Cys peroxiredoxin functions in the NAD(P)H-Trx system in both cytosol and plastid (Lemaire et al., 2004). Ferredoxins have also been identified as putative Trx h targets, in agreement with previous reports (Hagglund et al., 2008; Marchand et al., 2004). However, ferredoxin functions predominantly in chloroplasts with Trx isoforms f, m, x, and y [22]. Thus, the interaction with Trx h (predominantly cytosol, mitochondria, and endoplasmic reticulum) might not represent in planta situation. The loss of Trx subcellular specificity also occurs in other in vitro assays such as the capture method using Trx affinity columns (Yamazaki et al., 2004). To enhance the likelihood of identifying bona fide Trx h substrate, the subcellular localization of the putative proteins listed in Table 4-1 was checked. For proteins whose localization information is lacking, Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) was used to predict the localization. Proteins that reside in organelles other than where Trx h is located are likely to be false positives, rather than bona fide substrates. Those proteins were denoted by a star in Table 4-1. Isolation of a single cellular compartment for specified Trx interactions has been performed to address the issue of specificity (Lemaire et al., 2004).

Both large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase, phosphoribulokinase, and fructose-bisphosphate aldolase 1 were identified as Trx h targets in guard cell-enriched epidermal peels (Table 1). This is in line with studies in other plant systems showing that these enzymes are Trx targets (Balmer et al., 2004a; Bartsch et al., 2008; Marchand et al., 2006). In addition, subunit psaK of photosystem I reaction center was identified as a Trx h target (Balmer et al., 2006b). Similarly, we identified photosystem I subunit VII, i.e., psaC. Furthermore, a
novel protein containing 2Fe-2S iron-sulfur cluster binding domain was also identified here. These findings are in agreement with the notion that photosynthesis is highly redox-regulated and that this process is actively involved in stomatal function (Lawson, 2009).

**Discussion**

A great portion of the substrates revealed by this study were previously functionally characterized as Trx targets, demonstrating the reliability of this cysTMT method in Trx target identification. Additionally, previous studies using traditional methods usually identified 10-50 targets in one experiment [4]. The number identified in our study has been greatly increased, suggesting utility of this new multiplex technology. The combination of samples labeled with six cysTMT tags enhanced the probability in identifying low-abundance proteins, and enrichment of cysTMT-labeled peptides made it more feasible to identify Trx-reactive proteins. Moreover, the established Trx targets could contain one or two cysteine residues, forming intra- or inter-molecular disulfide bonds (Marchand et al., 2010; Montrichard et al., 2009). However, localization of the redox-sensitive cysteine residues is lacking in traditional methods such as trapping the potential targets with Trx mutant. In our method, Trx targeted cysteines were precisely localized in each target protein, due to the enrichment and identification of cysTMT labeled peptides. However, further investigation using biochemical and genetic tools is needed to determine the potential reaction mechanisms. For instance, analysis of target reduction upon Trx treatment and *in vivo* protein-protein interaction will improve our understanding of Trx-target mediated biological processes.
It should be noted that a little over 10% of the identified cysTMT peptides exhibited ratio changes opposite to that of expected Trx-targets (Table 4-1). These peptides and their associated proteins are deemed not to be Trx-targets. Although the reason is not known, we postulate that these proteins might be subjected to oxidization due to possible association with Trx-targets and/or irreversible oxidation might have taken place under control conditions. In addition, technical issues of incomplete alkylation might also contribute to false negatives. As mentioned before, an obvious challenge of in vitro Trx target analysis is the disruption of subcellular compartments, which may lead to ambiguity of identification of bona fide targets. Although Trx h is not solely localized in the cytoplasm [39] and its targets were found in the chloroplasts [7, 10, 27], the biological relevance of these findings deserve further investigation. More than 95% of chloroplast proteins are imported from the cytosol through the translocation complex in both the outer and inner chloroplast membrane (Shi et al, 2013). It will be interesting to see whether transport of Trx among organelles occurs.

In summary, this study showed the suitability of cysTMT in identification and multiplex quantification of cysteine modifications in discovering potential Trx targets. The six-plex cysTMT represents an isobaric mass tag system that specifically labels cysteines, providing unambiguous localization of cysteine modification and a convenient way to enrich cysteine-containing peptides. The reporter ions allow for quantitation of six samples in a single experiment. It is also notable that there are other applications of cysTMT in redox proteomics such as cysteine redox status monitoring (Parker et al., 2015) and mapping of S-nitrosylation sites (Murray et al., 2012). Therefore, more applications of cysTMT in redox proteomics can be expected in the future.
Table 4-1. Trx h targets identified from enriched guard cells using cystMT LC-MS/MS

<table>
<thead>
<tr>
<th>Accession</th>
<th>Protein Name</th>
<th>Peptides containing Trx h targeted cysteines</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>119952*</td>
<td>Redox Homeostasis (8)</td>
<td>AGAcSTcAGQIVK</td>
<td>1.50</td>
</tr>
<tr>
<td>3023740*</td>
<td>Ferredoxin</td>
<td>AGAcSScAGK</td>
<td>1.29</td>
</tr>
<tr>
<td>296514476</td>
<td>2-Cys peroxiredox</td>
<td>TLQALQYQVENPDEVcPAGWKPGK</td>
<td>1.57</td>
</tr>
<tr>
<td>75336180*</td>
<td>Peroxiredoxin Q, chloroplastic</td>
<td>GKPVVVFYFPADETPGcTK</td>
<td>1.30</td>
</tr>
<tr>
<td>297816586*</td>
<td>Peroxiredoxin-2E, chloroplastic</td>
<td>TILFAVPGAFTPTcSQK</td>
<td>1.57</td>
</tr>
<tr>
<td>79320786</td>
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<td>VILFGVPGAFTPTcSmK</td>
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</tr>
<tr>
<td>25552726</td>
<td>Ferredoxin-thioredoxin reductase, chloroplast</td>
<td>SGTYFcVDK</td>
<td>1.82</td>
</tr>
<tr>
<td>297809773*</td>
<td>Thioredoxin M2, chloroplastic-like isomor X2</td>
<td>ADGPVLVDFWAPWcGPcK</td>
<td>1.39</td>
</tr>
<tr>
<td>52354317</td>
<td>Photosynthesis (9)</td>
<td>DcGPmVLDALIK</td>
<td>1.53</td>
</tr>
<tr>
<td>1352767*</td>
<td>Ribulose bisphosphate carboxylase large chain</td>
<td>WSPELSSAcEVWK</td>
<td>1.27</td>
</tr>
<tr>
<td>266891*</td>
<td>Ribulose bisphosphate carboxylase small chain</td>
<td>QVQcISFIAYKPPSFTG</td>
<td>1.59</td>
</tr>
<tr>
<td>113201045*</td>
<td>Photosystem I subunit VII</td>
<td>TEDcVGcK</td>
<td>1.53</td>
</tr>
<tr>
<td>115778*</td>
<td>Chlorophyll a-b binding protein 1, chloroplastic</td>
<td>ELEVIHcR</td>
<td>1.33</td>
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<tr>
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<td>Photosystem II subunit O-2</td>
<td>FcFEPTSFTVK</td>
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Note: Lowercases c and m in the peptide sequences represent cysTMT labeled cysteines and oxidized methionine, respectively.
Figure 4-1. Scheme for identification of Trx h targets using cysTMT labeling and tandem MS. The ordered chemical reactions for a Trx target in the control (upper panel) and Trx h treated (lower panel) samples are presented. Protein extracted from *B. napus* epidermal peels was pre-oxidized by incubation with air (O_2) overnight [20]. Recombinant Trx h was activated by incubation with DTT for 30 min at room temperature, followed by binding to the nickel column and being washed thoroughly to remove DTT. Oxidized protein extract was incubated with or without Trx h for 1 h at room temperature. IAM was then added to the mixture at a final concentration of 100 mM for protein alkylation to block remaining free thiol groups. The oxidized cysteines were reduced and labeled with cysTMT tags, followed by LC-MS/MS. The MS spectra represents the theoretical distribution of the cysTMT tags at the low m/z region, where the intensities of 126, 127, and 128 from the control group are higher than those of 129, 130, and 131 from the Trx treated groups. The insert indicates the opposite trend for Trx h itself.
Figure 4-2. Thx h reduces air-oxidized proteins from *B. napus* epidermal guard cells. DNTB assay of free thiols under different treatment. Data are presented as means with standard errors of three biological replicates. Inserted $p$ values indicate whether the differences among the groups are statistically significant using $t$-test.
Figure 4-3. Annotated mass spectrum of a representative cysteine-containing peptide showing differential redox status from control and treated samples. The MS/MS ions used to identify the peptide were labeled, and the intensity of individual cysTMT peak for quantification was inserted in the upper-left corner. This peptide was used to identify the Photosystem I reaction center subunit N as a putative Trx target.
Figure 4-4. Classification of the 80 putative Trx h targets into molecular functions.
CHAPTER 5
CONCLUSIONS AND PERSPECTIVE

MPK cascades are highly conserved signaling pathways that respond to environmental cues. Evidence in this study showed that BnMPK4 is activated in vivo by H₂O₂ and ABA. Transient expression of a constitutively active MPK4 construct causes H₂O₂ production and cell death in N. benthamiana leaves, indicating a positive role of MPK4 in ROS production. Thus, it is likely that MPK4 and ROS form a positive loop in plant stress signaling. Since the in vitro biochemical analysis revealed that the activity of recombinant MPK4 is not affected directly by H₂O₂, another mechanism to stop the amplification of the loop should be in place for proper cellular activities. The finding that phosphorylation of MPK4 caused protein aggregation in the presence of H₂O₂ provided a clue that control of protein turnover maybe responsible for the negative feedback regulation of the MPK4 cascade and thus the ROS homeostasis.

Advances in MS have made it possible to identify and quantify thousands of proteins in one experiment. Notably, it also allows for the identification of interacting proteins of interest to answer biological questions. When coupled with immunoprecipitation, the presented study found an array of proteins that are associated with MPK4. The majority of the putative MPK4 target candidates are involved in stress responses and plant growth, correlating with the previous findings that MPK4 is a stress-responsive kinase and that the mpk4 mutant is retarded in growth. The newly identified MPK4-interacting proteins need to be validated by other analytical tools before further investigations are made to reveal the physiological relevance of the interaction. The analysis of transgenic plants overexpressing MPK4 also shed new light on the role of MPK4 in plant immunity. The increase in pathogen resistance was correlated with a
suppression of JA signaling in the transgenic lines. In addition, the transgenic lines were more sensitive to flg22-triggered ROS burst in guard cells, which resulted in an enhanced stomatal closure.

While the power of MS in protein identification and phosphorylation mapping was used in MPK4 substrate identification, the quantification feature of MS was employed in the identification of Trx h targets. The six-plex cysTMT technology enabled high throughput and decreased experimental variation, providing three replicates of pairwise comparison in a single LC-MS run. In addition, cysteine-containing peptides were enriched, and the redox status of those cysteine-containing peptides can be quantified accurately. Many interesting guard cell proteins were identified as potential Trx h substrates with this quantitative proteomics workflow.
(i) **Screening techniques.** Several large-scale techniques have been used for identifying MAPK substrates. In Y2H, a MAPK is used as a bait to screen a plant cDNA library of interest. One significant drawback of Y2H is that a high false positive rate is often observed. For microarray based screening, a MAPK is incubated with protein microarrays in the presence of γ-32P; thus a subsequent quantitative analysis of transphosphorylation indicates a MAPK substrate on the microarray. This method is inherently limited by the number of proteins present on the array. Peptide library screening, similar to the protein microarray approach in the *in vivo* transphosphorylation reaction, uses peptides instead of proteins during the incubation. The resulting peptides are subject to proteomics assay to identify the phosphorylation sites and to infer the putative substrates. Both phosphoproteomics and AP-MS are MS-based screening methods. In the former, the differences in global phosphorylation level are compared between plants with or without MAPK activation using quantitative phosphoproteomics, and proteins that show significantly higher phosphorylation in MAPK-activated plants are considered as putative substrates. In the latter method, interacting proteins with the kinase of interest are co-purified from plant extract by affinity binding and then identified by proteomics. In both methods, proteins indirectly associated with MAPKs could be identified. In the ATP analog-sensitive kinase assay, kinase substrates are labeled with ATP analog such as ATP-γ-S, resulting in thiophosphorylation of substrates. This allows for immunoaffinity purification of the thiophosphopeptides and identification of the substrates by MS. In all screening method, follow-up validation work is essential to establish a MAPK-substrate relationship.

(ii) **Validation techniques.** A variety of methods are available to validate the physical interaction between a MAPK and a substrate at different levels, such as *in vitro*, *in planta*, and *in vivo*. An *in vitro* kinase assay is commonly used to determine whether a MAPK phosphorylates a substrate in a reaction tube. In this assay, the MAPK could be purified from a heterogeneous expression system such as *E. coli*, a transient expression system such as plant protoplasts and infiltrated *N. benthamiana* leaves, or a whole plant system such as wild type and transgenic plants. The putative substrate is typically purified from a recombinant expression system such as *E. coli*. The *in vitro* kinase assay produces phosphorylated substrates, the sites of which are typically mapped using MS. The resulting information provides basis to mutate the putative phosphorylation sites to determine the relevance of individual sites *in vitro* and *in vivo*. The *in vivo* interaction can be revealed by evidence such as same sub-cellular localization (co-localization), complex formation within the cell (co-IP), and physical contact of the proteins (BiFC and FRER). If the investigation is performed in a plant system other than where the native MAPK and substrate are interacts, such as in protoplasts and infiltrated *N. benthamiana* leaves, it is referred to as *in planta* interaction.
LIST OF REFERENCES


Silva-Sanchez C, Li HY, Chen S. 2015. Recent advances and challenges in plant phosphoproteomics. Proteomics 15, 1127-1141.


BIOGRAPHICAL SKETCH

Tong Zhang was born in the city of Huaibei, China, where he started his early education and completed his high school. His academic achievements began as a marketing major at the Northeast Agriculture University, Harbin, China in 2004. His interests turned into natural sciences, and he graduated with a Bachelor of Science in Biology in 2008 and a Master of Science in Botany in 2011.

Tong enrolled at the University of Florida in 2011 for doctoral work in the Department of Biology, concentrating on the application of mass spectrometry in the field of plant cell signaling. For five years, he conducted researches in a variety of areas in plant signaling and regulation. He also mentored students at different levels including higher school, undergraduate and graduate students. He received his Ph.D. from the University of Florida in the spring of 2016.