EIN MOLEKULARER MECHANISMUS DES "PRIMINGS" IN *ARABIDOPSIS THALIANA*

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A MOLECULAR MECHANISM OF PRIMING IN *ARABIDOPSIS THALIANA*

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"Art and science have their meeting point in method."
E. Bulwer-Lytton (1803 - 1873)

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ABSTRACT

In plants and animals acquired immunity to biotic and abiotic stress is associated with priming of cells for faster and stronger activation of defense responses. It has been hypothesized that cell priming involves accumulation of latent signaling components that are inactive until challenge exposure to stress. However, the identity of such signaling components has remained elusive. Here I show using various chemical compounds or inoculation with avirulent bacterial pathogens, that induction of the primed state of *Arabidopsis thaliana,* requires accumulation of mRNA and inactive protein of mitogen-activated protein kinases (MPK) 3 and MPK6. Only upon challenge exposure to biotic or abiotic stress, MPK3/MPK6 enzymes were strongly activated in primed plants, resulting in enhanced defense gene expression and manifestation of acquired immunity. Strong elicitation of stress-induced MPK3/MPK6 activity is also seen in the constitutive priming mutant *edr1*, while it was attenuated in the priming-deficient *npr1* mutant. Moreover, priming of defense gene expression and acquired immunity were lost in *mpk3* and *mpk6* mutant plants. My findings unveiled for the first time that pre-stress deposition of the signaling components MPK3 and MPK6 is a critical step in priming plants for potentiated defense responses. In addition, I conducted and validated a proteome-wide analysis of phosphorylated proteins to identify novel candidate proteins involved in the priming mechanism in *Arabidopsis*. A thorough understanding of the molecular mechanism of priming in *Arabidopsis* will contribute to modern pest management in the field and likely lead to new discoveries in non-plant research.

ABBREVIATIONS

3-OH-BA	3-Hydroxybenzoic acid
4CL	4-Coumarate:CoA ligase
4-Cl-SA	4-Chlorosalicylic acid
$Al(OH)_3$	Aluminium hydroxide
ANK	Ankyrin repeat
avr	avirulence
BTB	Broad-Complex, Tramtrack, Bric-à-brac
BTH	Benzothiadiazole S-methyl ester
C4H	Cinnamic acid 4-hydroxylase
EDR1	Enhanced disease resistance 1
ERK2	Extracellular signal-regulated kinase 2
ET	Ethylene
Flg22	Flagellin 22
Hpt	Hours post treatment
HR	Hypersensitive response
IEF	Isoelectric focusing
INA	2,6-dichloroisonicotinic acid
IPG	Immobilized pH gradient
ISR	Induced systemic resistance
JA	Jasmonic acid
LRR	Leucine-rich repeat
MALDI	Matrix-assisted laser desorption/ionization
MAMP	Microbe-associated molecular pattern
MAPK/MPK	Mitogen-activated protein kinase
MAPKK /MKK	MAPK kinase
MAPKKK/MEKK	MAPKK kinase

MBP	Myelin basic protein
MeSA	Methyl-salicylic acid
MOAC	Metal oxide affinity chromatography
MS	Mass spectrometry
NahG	Naphthalene (Salicylate) hydroxylase Gene
NB	Nucleotide binding
NIM1	Non-inducible immunity 1
NLS	Nuclear localization signal
NPR	Non-expressor of PR genes
PAGE	Polyacrylamide gel electrophoresis
PAL	Phenylalanine ammonium lyase
PAMP	Pathogen-associated molecular pattern
pI	Isoelectric point
PMF	Peptide mass fingerprint
POZ	Poxvirus, Zinc finger
PR	Pathogenesis-related
PRR	Pattern recognition receptors
Psm	*Pseudomonas syringae* pv. *maculicola*
Pst	*Pseudomonas syringae* pv. *tomato*
R gene	Resistance gene
ROS	Reactive oxygen species
RT	Room temperature
RT-qPCR	Real-time quantitative polymerase chain reaction
SA	Salicylic acid
SABP2	Salicylic acid binding protein 2
SAI1	Salicylic acid insensitive 1
SAMT1	Salicylic acid methyl transferase 1
SAR	Systemic acquired resistance

SDS	Sodium dodecyl sulfate
SID2	Salicylic acid induction deficient 2
TMV	Tobacco mosaic virus
WB	Western blotting
WP	Wettable powder

INTRODUCTION

To survive, all living organisms are in a constant battle against various biotic and abiotic stress cues. To cope with different types of stress, plants and animals are equipped with multiple layers of defense. Preformed physical barriers and secondary toxic metabolites form a passive first line of defense to fend off potential pathogens or to protect organisms against abiotic stress. Unlike animals, plants cannot move to escape environmental challenges and lack mobile defense-associated cells. Thus, plants in addition to a number of pre-existing barriers also depend on inducible cellular defense mechanisms to effectively ward off stress.

Basal Plant Defense

Plants are immune to the majority of potential harmful microorganisms due to the existence of their passive first defensive barrier. Preexisting physical and chemical defensive structures that hinder the advance of a pathogen is provided by waxy cuticular layers covering the epidermal cells, thick cell walls, and antimicrobial compounds. So-called "non-host pathogens" are unable to overcome this first layer of defense and, thus, lack the ability to initiate infection on most plant species resulting in "non-host resistance" (Ellis, 2006).

Besides the preformed defensive structures, potential host plants are capable of activating a battery of inducible defenses. Recognition of general elicitors of microorganisms, so-called microbe- or pathogen-associated molecular patterns (MAMP/PAMP), such as flagellin, chitin, ergosterol and lipopolysaccharides, activate an innate immune response in plants (Gómez-Gómez and Boller, 2002). Perception of such micro-bial determinants through transmembrane pattern-recognition receptors (PRR) triggers, amongst others, activation of mitogen- activated protein kinase (MAPK)-dependent signaling cascades (Asai et al., 2002), ultimately stimulating early induced defense responses, like e.g. ethylene emission and the production of reactive oxygen species (ROS) (Jones and Dangl, 2006).

An accelerated and amplified defense response is triggered upon a plant's recognition of more specific pathogen-encoded effector molecules by using nucleotide binding and leucine-rich repeat domain (NB-LRR) proteins. Governed by specific interaction between pathogen effector gene loci and alleles of the corresponding plant disease resistance (R) locus so-called "gene-for-gene resistance", plants often activate the locally effective hypersensitive response (HR), which includes rapid cell death of infected tissue to slow down the pathogens and prevent it from spreading further (Dangl and Jones, 2001). In addition to the pathogen-specific immune response, invaders face a second line of inducible acquired immune responses. In plant parts distant from the site of primary infection, systemic responses establish an enhanced defense capacity against subsequent infections.

Induced Resistance

Three prominent induced resistance phenomena have been described: β-aminobutyric acid (BABA) induced resistance (Zimmerli et al., 2000; Ton et al., 2005), root-colonizing rhizobacteria-mediated induced systemic resistance (ISR) (van Loon et al., 1998), and pathogen-induced systemic acquired resistance (SAR)(Durrant and Dong, 2004). Whereas ISR depends on the plant hormones jasmonic acid (JA) and ethylene (ET) (Pieterse et al., 1998), SAR is mediated by salicylic acid (SA). SAR has been increasingly well studied ever since its discovery at the beginning of the 20th century (Beauverie, 1901).

Upon infection with a pathogen, SAR can be induced at the site of infection as well as in uninfected distal tissues. This biologically induced resistance in systemic tissue has been shown to be effective in many plant species. The attained state of resistance is long-lasting and effective not only against a broad spectrum of pathogens, including pathogenic bacteria, fungi, oomycetes and viruses (Ryals et al., 1996; Sticher et al., 1997; Durrant and Dong, 2004) but also protects plants against abiotic stress (Janda et al., 1999; Senaratna et al., 2000). SAR is associated with activation of a large set of pathogenesis-related (*PR*) genes in the local and, to a quantitative lesser extent, in systemic tissues (Ward et al., 1991; Maleck et al., 2000). It is generally thought that SAR is the result of concerted action of proteins encoded by many *PR* genes and, thus, activation of *PR* genes serves as a powerful molecular marker for the onset of SAR (Ryals et al., 1996). It has long been assumed that the plant hormone SA functions as a signaling molecule in SAR. Malamy et al. (1990) reported elevated endogenous SA levels in both local and systemic tissues after tobacco mosaic virus (TMV) infection of tobacco plants, which correlated with the induction of *PR* genes. Similarly, it was found that levels of SA in the phloem sap of cucumber plants rose after tobacco necrosis virus (TNV) infection or upon inoculation with the fungal pathogen *Colletotrichum lagenarium* (Métraux et al., 1990). Further support for the involvement of SA in SAR came from the finding that exogenous application of SA or the synthetic SA analogs 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole S-methyl ester (BTH) could induce the same set of *PR* genes (Ward et al., 1991; Friedrich et al., 1996; Görlach et al., 1996; Lawton et al., 1996). Transgenic plants carrying the bacterial *nahG* transgene, encoding a bacterial SA hydroxylase that converts SA to catechol, cannot accumulate high levels of SA, fail to express *PR* genes, and do not express SAR (Gaffney et al., 1993; Delaney et al., 1994; Lawton et al., 1995). Besides these transgenic plants, several *Arabidopsis thaliana* mutants with defects in SA-signaling (Glazebrook et al., 1996; Jirage et al., 1999; Nawrath et al., 2002) and biosynthesis (Nawrath and Métraux, 1999) were also described. *Arabidopsis* plants were shown to synthesize SA from chorismate via isochorismate during defense responses (Strawn et al., 2007). The *SALICYLIC-ACID-INDUCTION DEFICIENT 2* (*SID2*) gene encodes a chloroplast-localized isochorismate syn-

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thase catalyzing the conversion of chorismate to isochorismate (Strawn et al., 2007). Mutant *sid2* plants exhibit reduced SA accumulation in response to pathogens, reduced *PR* gene expression and enhanced susceptibility to pathogens (Wildermuth et al., 2001). Recently, it was reported that upon activation of defense responses in the primary infected tissue SA is converted to the volatile ester methyl-SA (MeSA) by SA methyl transferase (SAMT1), thereby generating a SAR mobile signal transported through the phloem. In the systemic tissue, SA-binding protein 2 (SABP2) is required to convert MeSA back to free SA. Silencing of either *SAMT1* or *SABP2* compromised SAR in tobacco plants (Park et al., 2007). Together these data confirm SA as a key signaling molecule in plant defense in general and that SA is required for activation of SAR.

Priming as a Mechanism of Induced Resistance

Over the past decade, all prominent induced resistance phenomena have been associated with the enhanced ability to ward off pathogen attack and resist abiotic stress by more rapid and/or stronger activation of cellular defense responses (Conrath et al., 2006). The physiological condition in which plants are able to stronger and/or more rapidly mount defense responses to biotic or abiotic stress is called the "primed" state of the plant (Conrath et al., 2001). Priming by infection with pathogens or by colonization of the roots with beneficial microbes induces the primed state in distal untreated tissue (van Wees et al., 1999; Kohler et al., 2002). Additionally, various natural and synthetic compounds (SA, BABA, BTH, strobilurin fungicides) have been shown to be able to induce the primed state in plant cell cultures and intact plants (Thulke and Conrath, 1998; Zimmerli et al., 2000; Herms et al., 2002; Kohler et al., 2002).

Biological induction of SAR in plants is not only associated with direct activation of *PR* genes, SAR-induced SA accumulation also primes systemic organs for augmented activation of defense responses (Conrath et al., 2006). However, the potentiated induction of defense responses such as augmented expression of defense-related genes only becomes apparent upon challenge treatment of primed plants. For example, biological induction of priming in *Arabidopsis* by local infection with avirulent *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 triggers accumulation of the endogenous signaling hormone SA, which mediates activation of *PR-1, -2,* and *-5 (Durrant and Dong, 2004)*. In addition, after subsequent pathogen challenge, primed *Arabidopsis* displays an augmented expression of defense-related phenylalanine ammonia-lyase (*PAL*) and *PR-1* genes (Kohler et al., 2002). Priming for potentiated *PAL* expression could be evoked by pretreatment with low doses of the synthetic SAR inducer BTH, which resulted in augmented accumulation of *PAL* mRNA after infection with virulent *Pst* DC3000 (Kohler et al., 2002). Similarly, priming for enhanced defense gene expression was also observed in primed plants that were challenged by wounding with forceps or infiltrated

with water on their leaves. Another study indicating priming plays a role in SAR signaling used transgenic tobacco plants carrying chimeric *Asparagus officinalis PR-1::uidA* and *PAL-3::uidA* reporter gene constructs (Mur et al., 1996). After pretreated with SA, these plants displayed greatly enhanced induction of the *PR-1* and *PAL* genes upon pathogen challenge inoculation although the reporter genes were not directly activated by SA treatment alone (Mur et al., 1996).

A Central Role for NPR1 in Induced Resistance

Several mutant screens have been performed to identify *Arabidopsis* mutants with defects in the SA-dependent SAR signal transduction pathway. Strikingly, three independent screens identified mutants that contain a mutation in the regulatory gene *NPR1/NIM1/SAI1 (nonexpressor of PR genes/non-inducible immunity/ salicylic acid-insensitive) (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997)*. Mutant *npr1* plants accumulate high levels of SA after infection, but fail to activate *PR* genes and are highly susceptible to a wide range of pathogens (Cao et al., 1994; Shah et al., 1997). Moreover, primed wild-type plants show potentiated expression of SAR-related genes after challenge inoculation, whereas in mutant *npr1* plants potentiation is lost (Conrath et al., 2002; Kohler et al., 2002). Thus, *NPR1* encodes an important positive regulator that transduces the SA signal in SAR and plays a key role in priming. Cloning of *NPR1* revealed two conserved protein-protein interaction domains within the NPR1 protein: an ankyrin repeat (ANK) domain and a Broad-Complex, Tramtrack, Bric-à-brac/Poxvirus, Zinc finger (BTB/POZ) domain (Bork, 1993; Aravind and Koonin, 1999). In addition, a nuclear localization signal (NLS), multiple conserved cysteine amino acid residues, and several putative phosphorylation sites were identified in NPR1 (Cao et al., 1997; Ryals et al., 1997; Kinkema et al., 2000; Mou et al., 2003).

In wild-type *Arabidopsis NPR1* is constitutively expressed and its mRNA levels increase two- to three-fold upon pathogen infection or treatment with SA (Cao et al., 1997). Transgenic *Arabidopsis* lines overexpressing NPR1 do not exhibit an obvious phenotype, but show enhanced induction of *PR* genes only upon pathogen infection. Accordingly, these plants show enhanced disease resistance that is dependent on NPR1 dosage (Cao et al., 1998). Overexpression of *Arabidopsis NPR1* in rice conferred enhanced resistance to the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* (Chern et al., 2001). Thus, by overexpressing the key regulator of SAR, plant immunity can be boosted in both monocots and dicots, suggesting that they share at least partly similar defense signaling pathways. NPR1 homologues were identified in several crop plants, such as apple, sugar beet, tobacco and tomato (Durrant and Dong, 2004; Malnoy et al., 2007).

The importance of SA and NPR1 in SAR has long been established (Durrant and Dong, 2004). But how is the SA signal transduced to NPR1? Overexpression of *NPR1* does not lead to constitutive *PR* gene expression in the absence of SAR induction, indicating that NPR1 requires an activation step to be functional. Several studies have suggested that changes in endogenous SA levels after pathogen infection can affect the redox state of the cell (Chen et al., 1993; Vanacker et al., 2000). Together with the fact that NPR1 contains conserved cysteine residues that are often subject to redox regulation, Mou et al. (2003) investigated the possibility of redox-mediated activation of NPR1 to provide the missing link between accumulation of SA and activation of NPR1. It was demonstrated that monomeric NPR1 protein links together via intermolecular disulfide bonds at cysteine residues 82 and 216 to form a high molecular weight oligomeric complex (Mou et al., 2003). S-nitrosylation of cysteine residue 156, facilitated by S-nitrosoglutathione, promotes the multimeric state of the complex (Tada et al., 2008). Pathogen-induced SA accumulation or treatment with SAR inducers results in an early transient increase in cellular reduction potential followed by a rapid decrease in reduction potential (Mou et al., 2003). To counter the effect of S-nitrosylation of NPR1 upon pathogen infection, cytosolic thioredoxins catalyze the reduction of intermolecular disulfide bonds of NPR1 thereby facilitating the oligomer-to-monomer switch (Tada et al., 2008). This process precedes the activation of *PR* gene expression, suggesting that NPR1 monomerization is required for activation of *PR* genes. Indeed, solely monomeric NPR1 protein carrying an intact NLS is capable of being translocated into the nucleus to activate *PR* genes, whereas the high molecular weight oligomer is thought to be retained in the cytoplasm (Kinkema et al., 2000; Mou et al., 2003).

Plant Defense Regulation: Signal Interplay of MAPK Networks

In all eukaryotes, mitogen-activated protein kinase (MAPK) cascades transduce information of extracellular stimuli to intracellular responses. MAPK pathways, operate downstream of receptors or sensory signaling molecules and are involved in regulating growth, development, and responses to endogenous and environmental cues. A MAPK cascade typically consists of three functionally tiered protein kinases. Members of the family of MAPK kinase kinases (MAPKKK/MEKK) comprise the conserved entry points of MAPK cascades. In plants, MEKKs modulate activity of downstream MAPK kinase (MAPKK/MKK) through simultaneous phosphorylation of serine (S) and threonine (T) amino acid residues in the plant-specific consensus phosphorylation target site motif, -S/TxxxxxS/T-, within the activation loop. In turn, activation of *Arabidopsis* MAPKs by MKKs is regulated by dual phosphorylation of the highly conserved T and tyrosine (Y) residues in a tripeptide motif (TxY, where x could be either glutamic acid (E) or aspartic acid (D)) located in the activation loop between subdomains VII and VIII of the kinase catalytic domain (Zhang and Klessig, 2001; Ichimura et al., 2002;

Hamel et al., 2006). Activated MAPKs then phosphorylate various targets, which often include transcription factors (Herskowitz, 1995). Though only very few substrates of plant MAPKs have been identified to date (Asai et al., 2002), the recent development of novel high-throughput methodology for the assessment of protein phosphorylation will likely accelerate the identification of MAPK substrates in the future (Feilner et al., 2005; Peck, 2006; Popescu et al., 2007; Ritsema et al., 2007).

Unbalancing a Tightly Regulated MAPK Signaling Network: Effects on Induced Resistance Responses

Accumulating evidence indicates that plants rapidly activate MAPK cascades when exposed to multiple biotic and abiotic stress stimuli (Ichimura et al., 2000; Zhang and Klessig, 2001). The identity, roles, and specificity of MAPK cascades in the regulation of diverse stress responses, and their link to defense mechanisms, are the focal points of intensive research. To date several *Arabidopsis* MPK signaling mutants have been described that carry defects in genes encoding components of the MAPK signaling network (Frye et al., 2001; Teige et al., 2004; Wang et al., 2007). Some of the mutations cause dramatic effects on induced resistance responses of plants. The *enhanced disease resistance 1* (*edr1*) mutant described by Frye and Innes (1998), was first identified in a mutational screen for *Arabidopsis* plants expressing elevated resistance to *P. syringae*. Resistance in *edr1* plants is even more effective against the fungal powdery mildew pathogen *Golovinomyces cichoracearum* (Frye and Innes, 1998). *Arabidopsis* mutants showing enhanced resistance to pathogens had previously been described, but those mutants display constitutively elevated levels of SA and constitutive *PR* gene expression (Glazebrook, 1999). These phenotypes are often associated with dwarfing of the plant and/or aberrant cell death (lesion formation on uninfected leaves). The *edr1* mutant exhibits none of the above traits (Frye and Innes, 1998). The augmented defense phenotype of *edr1* depends on SA and NPR1 (Frye et al., 2001). This latter result is significant because, it shows that disruption of the MAPKKK-encoding *EDR1* gene does not cause constitutive activation of SA-mediated defense pathways, but rather causes enhanced responsiveness to induction of those pathways by pathogens (Frye et al., 2001; van Hulten et al., 2006). Thus, *edr1* is a mutant that is constitutively primed.

Based on experiments with *Saccharomyces cerevisiae* the first hypothetical *Arabidopsis* MAPK cascade that was proposed in *Arabidopsis* consist of MEKK1, MKK1/MKK2, and MAPK4 (MPK4) (Ichimura et al., 1998). However, crosstalk between different MAPK cascades in abiotic stress signaling was reported leading separately to activation of MPK3, MPK4, and MPK6 (Ichimura et al., 2000; Kovtun et al., 2000; Miles et al., 2005). Specific pathogen-activated MAPK cascades have also been reported. For example, MEKK1, MKK4/MKK5, MPK3/MPK6 were demonstrated to function downstream of the LRR receptor kinase FLS2,

that was identified as the receptor for flagellin, a highly conserved peptide fragment of the bacterial flagellum (Gómez-Gómez and Boller, 2000; Asai et al., 2002; Chinchilla et al., 2006). Furthermore, the induction of camalexin, a phytoalexin that is active against necrotrophic fungal pathogens, such as *Botrytis cinerea*, was recently shown to be regulated by MPK3/MPK6 (Ren et al., 2008). As both MPK3/MPK6 are also activated by diverse abiotic and biotic stresses, these two proteins seem to be a part of the integrated stress-signaling network in plants by functioning as convergence nodes for many distinct signaling cascades in plant defense and stress responses. Besides the obvious post-translational regulation of enzyme activity of each of the three tiers of kinases, transcript regulation was reported for several MAPKs. However, until now the significance of this dual regulation remains unclear.

Here, the involvement of the MPK3/MPK6 proteins in the molecular mechanism of priming for induced resistance to biotic and abiotic stress is elucidated, and functional evidence is provided for the importance of the dual regulation of MAPKs in plant defense signaling. In addition, a proteome-wide analysis of phosphoproteins was performed to identify novel candidate proteins involved in the priming mechanism in *Arabidopsis.*

RESULTS

Biologically Active Salicylates Activate Expression of the *MPK3* Gene

Earlier results in the Conrath group indicated that the Elicitor-Responsive Mitogen-activated protein Kinase (ERMK) might play a role in priming of parsley suspension cells (Simonis, 2000). As a first step towards studying the molecular basis of priming a Basic Local Alignment and Search Tool (BLAST) sequence database search and sequence comparison have been performed. The parsley ERMK deduced amino acid sequence defined the MAPK 3 (MPK3) as its closest ortholog in *Arabidopsis thaliana*. The overall structure prediction based on deduced amino acid sequence of these two orthologous enzymes is highly conserved with a sequence similarity of 83% (Ligterink et al., 1997). To investigate whether *A. thaliana* MPK3 might be an important component of the priming/SAR mechanism, first it was assayed whether *MPK3* is induced by various compounds that are known to induce the primed state and acquired immunity in *A. thaliana*. We employed foliar application of biologically active (SA, 4-chloro-SA (4-Cl-SA) and BTH) and inactive (3-hydroxy-benzoic acid (3-OH-BA)) compounds that are structurally related to SA (Fig. 1A) (Conrath et al., 1995). Some of these compounds have been reported to mimic SA by acting as natural inducers of enhanced resistance to pathogens in *A. thaliana* and some other plant species (Ryals et al., 1996; Durrant and Dong, 2004). More recently, the biologically active salicylates (SA, 4-Cl-SA and BTH) have been shown to induce SAR and the primed state in *Arabidopsis* plants (Görlach et al., 1996; Kohler et al., 2002). The gene encoding MPK3 was activated only when plants were sprayed with these active compounds, but not upon spraying the biologically inactive, but structurally related, 3-OH-BA (Fig. 1B).
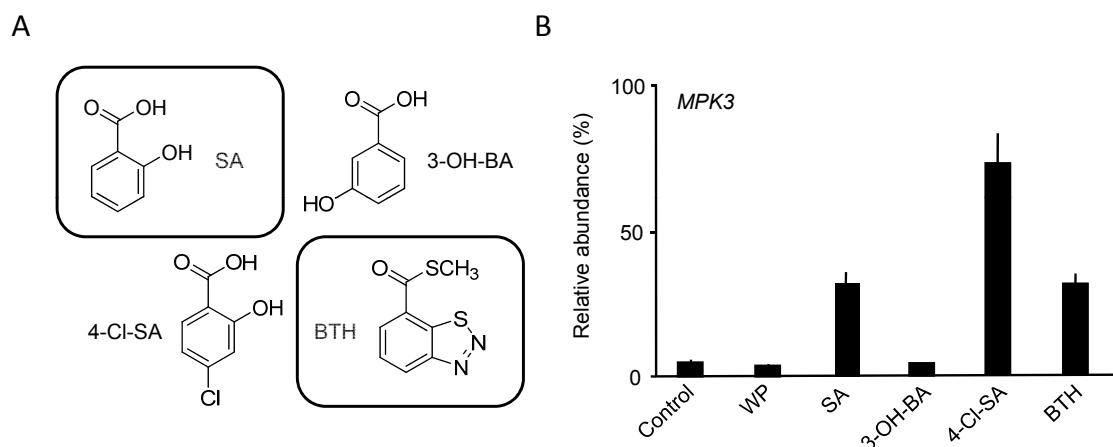

Figure 1: Chemical structure of SA analogs and their capacity to induce accumulation of MPK3 transcripts.
(A) Chemical structure of compounds that have been shown to be active (SA, 4-Cl-SA and BTH), or inactive (3-OH-BA), at the induction of the primed state in *Arabidopsis* (Kohler et al., 2002)**.**
(B) Leaves were harvested from *Arabidopsis* plants 3 days after spray treatment with 300μM SA, 300μM 3-OH-BA, 300μM 4-Cl-SA, or 100μM BTH. All these compounds were dissolved in a solution of wettable powder carrier (provided by Syngenta Corp.). Control plants were treated with wettable powder carrier (WP) only or left untreated (control). An aliquot of leaf tissue was used for RT-qPCR analysis to examine the relative abundance of transcripts for *MPK3* normalized to those for *ACTIN2*. The experiments were performed three times with similar results.

BTH Activates *MPK3/MPK6* Gene Expression and Induces MPK3/MPK6 Protein Accumulation

BTH is a synthetic chemical that mimics SA action in that it supplies resistance against a broad spectrum of diseases in a variety of crops (Beckers and Conrath, 2007); it is readily taken up by plants and is known to be stable in plants (Ryals et al., 1996). Therefore, BTH was used as the inducer of priming and SAR and to investigate whether MPK3 and its highly conserved homolog, MPK6, play a key role as signaling components involved in priming of *A. thaliana*. By doing so, it was found that during priming of *Arabidopsis* with 100µM BTH, transcripts for MPK3 and MPK6 accumulated in a biphasic manner over a basal level found in untreated plants (Fig. 2). MPK3 and MPK6 proteins also accumulated (Fig. 2) until 72 h after BTH treatment despite a decrease in transcript levels. However, the accumulation of MPK3 and MPK6 transcripts and proteins was not associated with dual phosphorylation of the TEY amino acid motif located in the activation loop of MPK3 and MPK6 (Fig. 2). Dual phosphorylation of the TEY motif located within the activation loop is required for enzyme activity (Ray and Sturgill, 1988; Zhang and Klessig, 1997, 2001). This result suggested that MPK3 and MPK6 accumulate in an inactive form in primed *Arabidopsis* plants. Therefore, these two proteins became prime candidates for cellular signaling enzymes that would contribute to the primed state in plants.
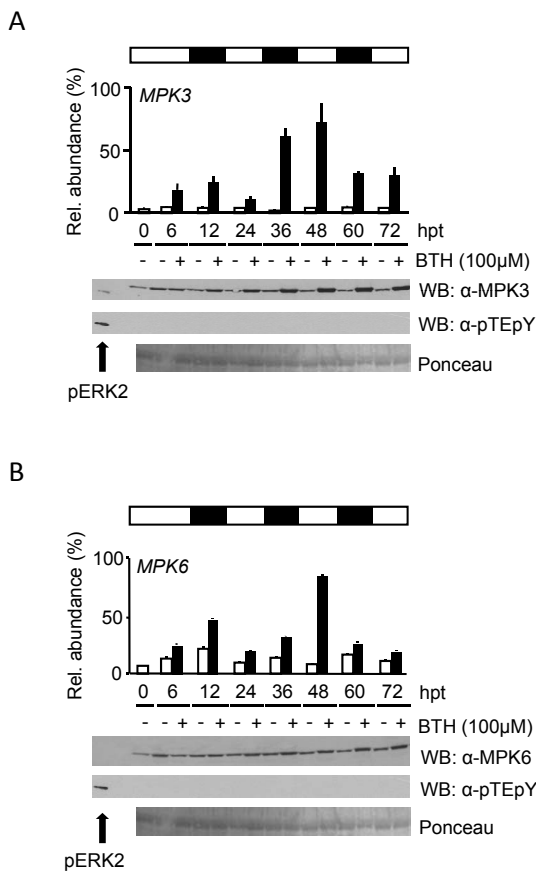

Figure 2: BTH induces accumulation of MPK3 and MPK6 transcripts and proteins but does not elicit TEY phosphorylation.
(A) Leaves were harvested at various times after treatment of plants with 100µM BTH (+) or a wettable powder carrier control (-). An aliquot of leaf tissue was used for RNA extraction and RT-qPCR analysis to examine the relative abundance of transcripts for *MPK3*. Another aliquot of leaves was used for protein extraction and SDS-PAGE followed by immunodetection of MPK3 and dual TEY phosphorylation with polyclonal antibodies. Immunodetection of a loaded doubly phosphorylated human ERK2 (pERK2) kinase served as positive control for blotting and immunodetection.
(B) Same experimental setup and analyses as in (A), but examining the relative transcript abundance of MPK6 and immunodetecting MPK6 and pTEpY. Prior to immunodetection, the blot was stained with Ponceau S for equal loading. The experiments were performed three times with similar results. Bars above diagrams give light/dark periods. Hpt, hours post treatment; WB, western blot.

Plants Primed by Pathogen Attack Systemically Accumulate Increased Levels of MPK3/MPK6 Proteins

The primed state and/or SAR can be induced in *Arabidopsis* by various treatments, such as inoculation of lower leaves with an avirulent bacterial pathogen (Kohler et al., 2002). To investigate whether priming by a localized infection with necrotizing pathogens increases the amount of latent MPK proteins in the distant parts of the plant, bona fide SAR experiments were conducted by inoculating three lower leaves with avirulent *Pseudomonas* bacteria. To that end two independent *P. syringae* strains (*Pseudomonas syringae* pv. *phaseolicola* (*Psp*) and *Pseudomonas syringae* pv. *tomato* (*Pst*)) expressing two different avirulence genes (*avrB* or *avrRpt2*) were used to induce the primed state. Systemic leaf tissue was harvested and analyzed two and three days after localized inoculation with the non-host pathogen *Psp* carrying the avirulence gene *avrB* (Dong et al., 1991). The abundance of MPK3 proteins increased in non-infected, systemic tissue (Fig. 3A). Likewise, upon local infection with the avirulent bacterial pathogen *Pst* harboring the avirulence gene *avrRpt2* MPK3 protein accumulated systemically (Fig. 3B). Immunodetection with the anti-phospho-TEY antibody suggested that the proteins remained in their non-phosphorylated state in the systemic, non-infected tissue (Fig 3B).
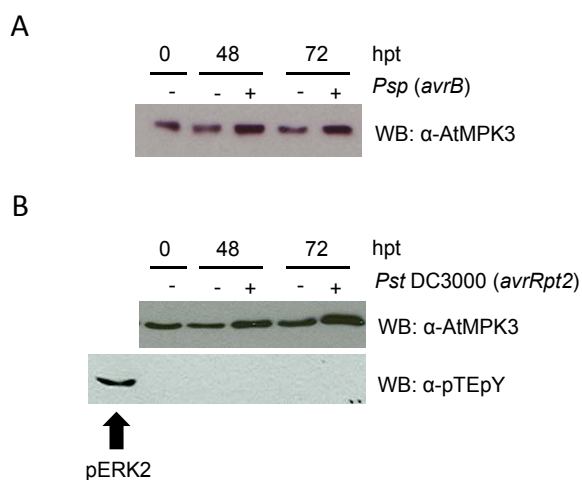

Figure 3: Systemic accumulation of MPK3/MPK6 during SAR.
(A) Three lower leaves of *Arabidopsis* wild-type plants were inoculated with *Psp* carrying the avirulence gene *avrB* ($2.5*10^7$ cfu/mL). At the times indicated, tissue was harvested from non-infected (systemic) leaves. The tissue was used for protein extraction and SDS-PAGE followed by immunodetection of MPK3 with polyclonal antibodies.
(B) Same experimental setup and analyses as in (A), but plants were inoculated with *Pst* DC3000 harboring the avirulence gene *avrRpt2* ($2.5*10^7$ cfu/mL). Immunodetection of MPK3 and pTEpY was done using polyclonal antibodies. The experiment was done twice with similar results. Hpt, hours post treatment; WB, western blot.

Augmented Phosphorylation and Activation of MPK3/MPK6 in BTH-Primed Plants Upon Stress Exposure

To elucidate whether MPK3/MPK6 might indeed display greater activity in primed and subsequently challenged *Arabidopsis* plants, primed leaves with enhanced levels of MPK3/MPK6 (Fig. 2) were exposed to stress. In the first instance, simple infiltration of tap water was used as the abiotic stress after the priming treatment since this stimulus elicits a cell collapse response that resembles the hypersensitive defense reaction already in the absence of avirulent pathogens (Roebuck et al., 1978) and is attended by defense gene expression (Young et al., 1996; Kohler et al., 2002; Beckers and Conrath, 2007). As shown in Figure 4A, after

water infiltration the levels of both MPK3/MPK6 further increased slightly until the 30-minute time point post infiltration in both primed and non-primed *Arabidopsis* leaves, with higher levels of MPK3/MPK6 being present in primed leaf tissue (Fig. 4A). The slight increase in MPK3/MPK6 levels after water infiltration may be due to infiltration-induced accumulation of transcripts for both enzymes. Transiently induced dual phosphorylation of the TEY activation site motif in MPK3/MPK6 upon infiltration was found in non-primed and primed leaves (Fig. 4A). In both types of leaves, dual TEY phosphorylation was strongest at the 10-minute time point after infiltration, but it was more intense and lasted longer in primed than in non-primed plants (Fig. 4A). The latter observation was especially true for MPK3 (Fig. 4A). Concomitantly, the enhanced dual TEY phosphorylation was in close correlation with augmented in-gel kinase activity of MPK6 and, particularly, MPK3 in primed plants (Fig. 4B).
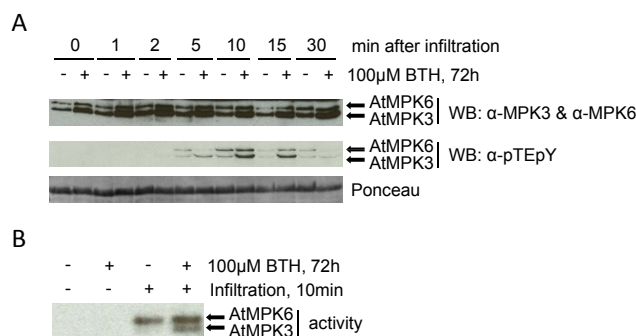

Figure 4: Dual TEY phosphorylation of MPK3/MPK6 and MPK activity are enhanced in primed leaves after their infiltration with water.
(A) Plants were primed with 100µM BTH (+) or treated with wettable powder carrier (-) for 3 days. Leaves were infiltrated with water, harvested at the times indicated, and analyzed for accumulation of MPK3, MPK6, and dual phosphorylation of the TEY motif using polyclonal antibodies.
(B) At the 10-minute-time point after infiltration, non-primed and BTH-primed leaves were analyzed for MPK enzyme activity by in-gel kinase assay with myelin-basic protein as the substrate. The experiments were done three times with similar results.

Enhanced Phosphorylation of MPK3 and MPK6 in Primed Plants after Challenge Infection

To investigate whether MPK3 and MPK6 indeed display quantitatively greater activity in primed and subsequently challenged *Arabidopsis* plants, BTH-treated leaves with enhanced levels of MPK3 and MPK6 (Fig. 2) were exposed to stress. After BTH-treatment leaves were dip-inoculated with virulent *Ps* pv. *maculicola* strain ES4326 (Dong et al., 1991). At various times after infection, an aliquot of leaves was analyzed for dual TEY phosphorylation. As is shown in Figure 5, activation-associated phosphorylation of MPK3 and MPK6 was induced upon bacterial infection of non-primed and BTH-primed leaves. In both types of leaves, dual TEY phosphorylation of MPK3 and MPK6 was strongest at the 30-minute time point after infection, but for MPK3 it was more intense and lasted longer in primed than in non-primed plants (Fig. 5).
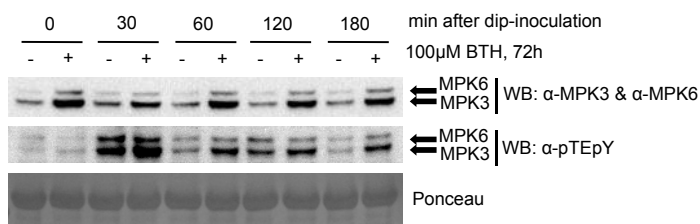
Figure 5: Dual TEY Phosphorylation is Enhanced in Primed Leaves after Dip-Inoculation with Bacteria.
Plants were treated with 100μM BTH (+) or wettable powder carrier (-) for 3 days. Leaves were then challenged by dipping into a suspension of *Ps* pv. *maculicola* strain ES4326 (5 x 10^8cfu/ml) in 10mM $MgCl_2$ containing 0.01% silwet L-77 (time point zero), harvested at the times indicated, and analyzed for dual phosphorylation of the TEY motif by SDS-PAGE, western blotting, and immunodetection with polyclonal antibodies.

Absence of Priming in *mpk3* and *mpk6* Mutants

The above results pointed to MPK3/MPK6 as possible key players in the mechanism of priming for the enhanced stress response in *Arabidopsis*. To elucidate whether the two enzymes indeed are crucial for priming, and to provide genetic evidence for this, knockout mutants and transgenic plants were analyzed. The expression of two genes, one encoding PAL1 and another one encoding PR1 were chosen as the markers for the activation of the plant defense response. These two genes were selected as their activation was known to be augmented in primed and subsequently challenged wild-type plants (Kohler et al., 2002) and *PAL1* gene activation is known to be regulated by MPK3/MPK6 (Tena et al., 2001). In addition, the PAL enzyme plays a rate-limiting role in the phenylpropanoid pathway with important functions in the overall plant defense response (Hahlbrock and Scheel, 1989), and *PR1* is widely considered as a marker gene for SAR in plants (Ryals et al., 1996; van Loon et al., 1998; Durrant and Dong, 2004). In wild-type *Arabidopsis*, priming with 100μM BTH did not activate the *PAL1* gene (Fig. 6A, B) and only slightly induced *PR1* (Fig. 6C). Infiltration of water into leaves elicited some *PAL1* expression (Fig. 6A, B) but didn't activate *PR1* (Fig. 6C). However, when the plants have been primed with BTH for 3 days and then stimulated on their leaves by water infiltration, there was boosted expression of both *PAL1* (Fig. 6A, B) and *PR1* (Fig. 6C).

When compared to wild-type plants, an *mpk3* deletion mutant (Li et al., 2001; Miles et al., 2005) (Fig. 6A) and an *mpk3* T-DNA knockout plant (Fig. 6B) were only slightly affected in *PAL1* activation by infiltration alone (Fig. 6A, B). Similarly, in *mpk3 PR1* expression was not altered by water infiltration (Fig. 6C). However, in contrast to the wild type, in the two *mpk3* mutants, the priming-mediated potentiation of infiltration-induced defense gene expression was absent for *PAL1* (Fig. 6A, B) and reduced for *PR1* (Fig. 6C).

Similar observations were made when the expression of two other genes in the phenylpropanoid pathway, 4-coumarate:CoA ligase (*4CL*) and cinnamic acid 4-hydroxylase (*C4H*), were assayed in wild-type and *mpk3* plants (Fig. 6D, E).
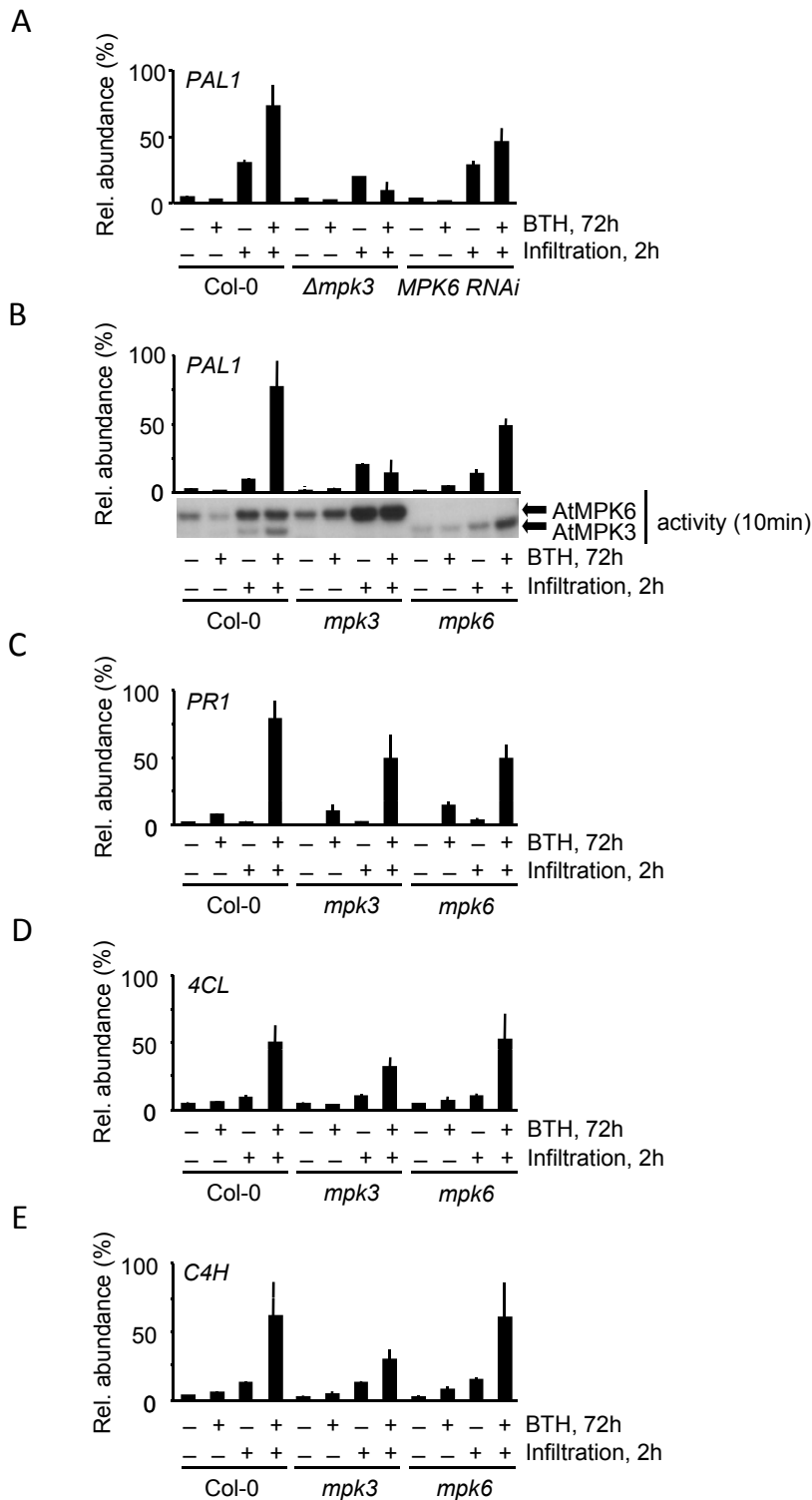
14

Figure 6: Absence or presence of priming for potentiated defense gene activation in mpk mutants.
(A) Wild-type (Col-0) plants, an *mpk3* deletion mutant (*Δmpk3*) and *MPK6* RNAi-silenced plants were pretreated with wettable powder carrier or 100μM BTH. Leaves were then left untreated or infiltrated with tap water and assayed for *PAL1* expression after 2 hours.
(B) Same experimental setup as in (A), but using *mpk3* and *mpk6* T-DNA insertion mutants, assaying *PAL1* expression after 2h and determining in-gel MPK activity 10 min after infiltration.
(C) Same as in (B), but assaying expression of *PR1* after 24h.
(D) and **(E)** Same setup as in (A), but analyzing expression of *4CL* and *C4H*.

Similar to the *mpk3* mutants, *PAL1* activation by infiltration alone was unaffected in non-primed *Arabidopsis* plants in which the expression of *MPK6* was repressed by RNA interference (RNAi) (Miles et al., 2005) (Fig. 6A), and in an *mpk6* T-DNA insertion mutant (Fig. 6B). In *mpk6*, *PR1* expression also was unchanged after water infiltration alone (Fig. 6C). However, in *MPK6* RNAi plants and the *mpk6* T-DNA mutant, priming-mediated enhancement of stress-induced *PAL1* expression was still observed, but to a lesser extent

than in the wild type (Fig. 6A, B). The same was true for the accumulation of *PR1* transcripts in *mpk6* (Fig. 6C). Similar findings were made when the expression of genes encoding 4CL and C4H was examined in these mutants (Fig. 6D, E). These results suggested that MPK3 is a major component in BTH-induced priming for potentiated defense gene activation in *Arabidopsis*, while MPK6 presumably serves a more minor role.

Accumulation of MPK3 Transcripts and Protein is Absent in *npr1*

From the above results, we concluded that pre-stress accumulation of MPK3 and MPK6 transcripts and protein primes *Arabidopsis* for potentiated defense gene activation resulting in enhanced resistance to abiotic stress. If this assumption holds true, one would expect that the BTH-induced accumulation of MPK3 and MPK6 transcripts and protein is lower or even absent in *Arabidopsis* plants with an impaired SAR response. To address this issue, we included the *Arabidopsis npr1* mutant in our experiments. Although this mutant is able to accumulate high endogenous levels of SA (Delaney et al., 1995) after pathogen attack, it does not express SA/BTH-induced priming for potentiated defense gene activation after *Pst* DC3000 infection, wounding with forceps, or infiltration of water into leaves (Kohler et al., 2002). Also, *npr1* does not display biologically or chemically induced resistance (Cao et al., 1994). Analysis of publicly available microarray data of a genomic approach to identify additional regulatory nodes in the transcriptional network of SAR (Wang et al., 2006) revealed that BTH induced the accumulation of *MPK3* and *MPK6* transcripts in wild-type *Arabidopsis* but didn't do so in the *npr1* mutant. In agreement with this, the BTH-induced pre-stress accumulation of MPK3 and MPK6 proteins and the greatly enhanced dual TEY phosphorylation of these two proteins after challenge exposure to stress which have been seen in the wild-type (Figs. 4-6), were greatly reduced in *npr1* (Fig. 7).

Enhanced Basal Levels of MPK3 Protein in *edr1* Correlate with Enhanced TEY Phosphorylation and Constitutive Priming for Augmented *PAL1* Expression

The enhanced disease resistance 1 (*edr1*) mutation of *Arabidopsis* confers resistance to the fungus *Golovinomyces cichoracearum*, the causal agent of powdery mildew of *Arabidopsis* (Frye and Innes, 1998). The *EDR1* gene was found to encode a MAP kinase kinase kinase, and various data suggest that EDR1 functions at the top of a MAP kinase cascade that negatively regulates SA-inducible defense responses (Frye and Innes, 1998; Frye et al., 2001). The enhanced resistance of the *edr1* mutant against powdery mildew correlated with a more rapid induction of defense responses, such as callose deposition and accumulation of autofluorescent compounds into the cell wall (Frye et al., 2001). It was hypothesized that the constitutively primed *edr1* mutant would display elevated basal levels of latent MPK3 proteins that leads to an enhanced
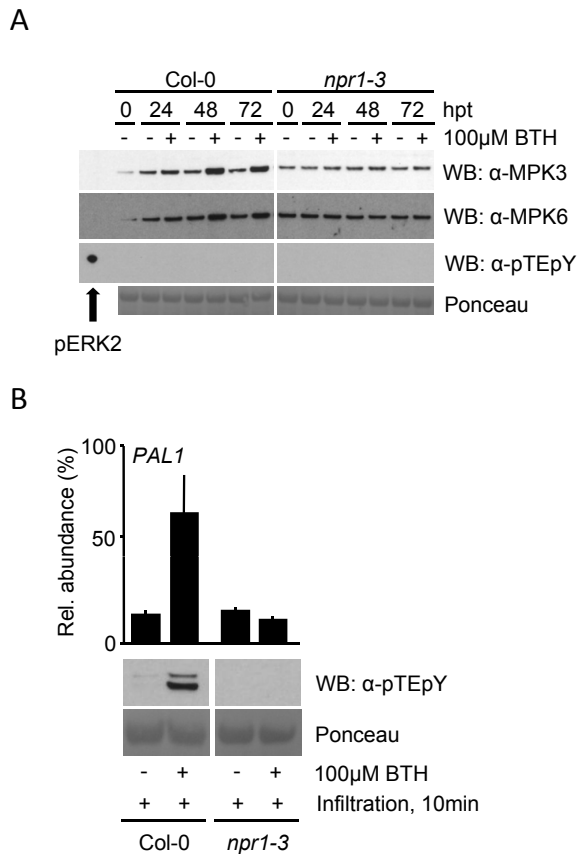
A

B

phosphorylation and activation of MPK3 upon a challenge treatment. To test this, primed and non-primed wild-type Col-0 and *edr1* plants were assayed for the presence of MPK3, the level of phosphorylated MPKs in challenged leaves as well as the activation of the *PAL1* gene (Fig. 8). Indeed, *edr1* mutant plants constitutively express slightly more non-phosphorylated MPK3 when compared to the wild type, although the abundance of MPK3 still increases upon treatment with BTH (Fig. 8). Interestingly, the dual phosphorylation of the TEY motif in *edr1* upon infiltration is much more pronounced in both primed and non-primed plants when compared to the wild type. The enhanced phosphorylation in *edr1* correlates with an enhanced induction of *PAL1*, even without prior induction of the primed state by treatment with BTH (Fig. 8).
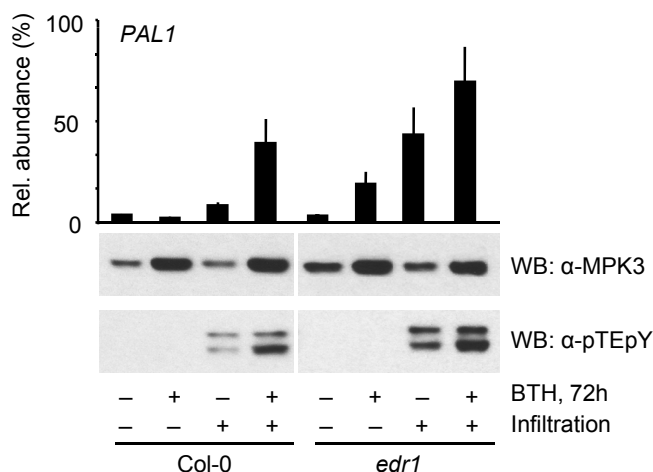
Chemically Induced Protection Against Bacterial Pathogens Is Impaired in *mpk3* and *mpk6*

Since *mpk3* and *mpk6* are affected in priming for the enhanced response to abiotic stress (Fig. 6) and because accumulation of MPK3 and MPK6 is impaired in *npr1* (Fig. 7), the question was addressed whether these signaling components might also play a role in the BTH-induced priming for the acquired resistance response to pathogens. Therefore, wild-type plants as well as *mpk3* and *mpk6* mutants were pretreated with BTH for 3 days and then infected by dipping the leaves into a suspension of virulent *Pst* DC3000. The dip-treatment enabled us to get rid of the infiltration stimulus that alone induced MPK phosphorylation (Fig. 4; 5) and activity (Fig. 6B) in *Arabidopsis*. *Pst*-infected leaves of BTH-pretreated and BTH non-pretreated wild-type, *mpk3* or *mpk6* plants were tested for bacterial multiplication by determining the titer of bacteria 4 days after *Pst* DC3000 infection and by examining inoculated leaves for disease symptoms. As shown in Figure 8, BTH-induced priming of wild-type plants resulted in lesser bacterial cells in infected leaves. The BTH-induced resistance to *Pst* DC3000 was reduced in the BTH-pretreated and subsequently infected *mpk3* mutant, and it was attenuated, to a lesser extent, in *mpk6* plants (Fig. 9).
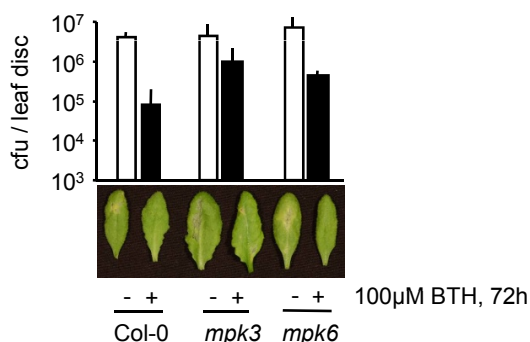
Figure 9: *mpk3* and *mpk6* mutants are attenuated in BTH-induced resistance to *Pst* DC3000.
Col-0, *mpk3*, and *mpk6* plants were pretreated with wettable powder carrier (-) or BTH (+). 3 days later, leaves of the plants were infected with *Pst* DC3000 (5×10^8 cfu/ml) by dip-inoculation. After another 4 days, leaf discs were harvested and analyzed for the presence of bacteria. Note that in some repeats of the experiment, induced resistance was more reduced in *mpk3*. Cfu, colony-forming units.

Pathogen-Induced Immunity against *Pseudomonas syringae* pv. *tomato* DC3000 is Compromised in *mpk3*

To further elucidate the role of MPK3/MPK6 in bona fide SAR, the question of whether these proteins regulate part of the pathogen-induced SAR response in *Arabidopsis* was addressed. Wild-type, *mpk3* and *mpk6* plants were first inoculated with *Pst* DC3000 carrying the plasmid-borne *avrRpt2* effector gene by infiltration of three lower leaves with bacterial suspension to induce bona fide SAR throughout the plant. Three days later, leaves were challenge inoculated with a bacterial suspension of virulent *Pst* DC3000. Bacterial *Pst* DC3000 titer was estimated 3 days after challenge infection in upper leaves. Concurrently to the results shown in Figure 9, pathogen-induced resistance against challenge inoculation with virulent *Pst* DC3000 was reduced in *mpk3* mutant plants (Fig. 10A). In wild-type plants and the *mpk6* mutant, SAR development was

preceded by more intense TEY phosphorylation of MPK3, but not MPK6, 2 hours after challenge infection of preinoculated plants (Fig. 10B).

A

B

Figure 10: Full expression of SAR is compromised in *mpk3*.
Three lower leaves of Col-0, *mpk3*, and *mpk6* plants were infiltrated with *Pst avrRpt2* ($5.0*10^5$ cfu/mL) in 10mM $MgCl_2$ or with 10mM $MgCl_2$.
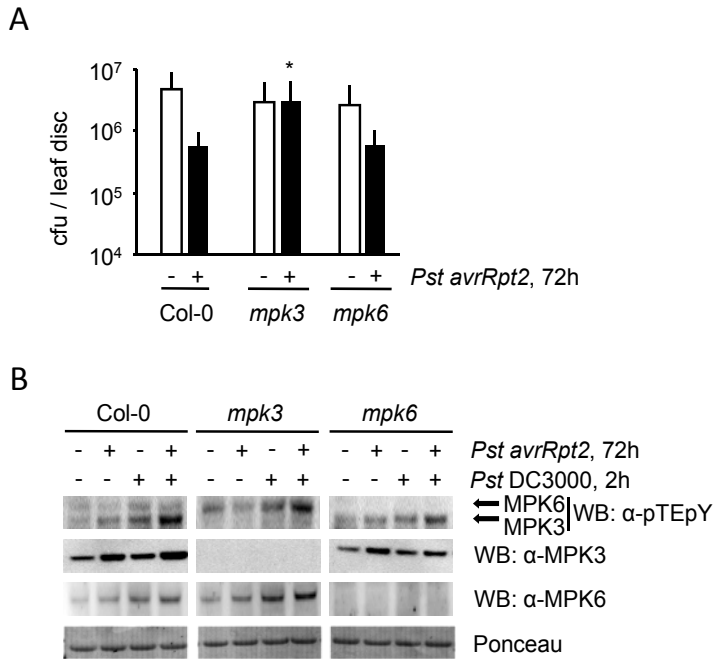**(A)** Three days later, leaves of the plants were infected with *Pst* DC3000 ($5.0*10^5$ cfu/ml) by pressure infiltration. After another 3 days, leaf discs were harvested from secondary infected leaves and analyzed for the presence of bacteria. Cfu, colony-forming units.
(B) Aliquots of leaves were harvested 2 h after dipping plants into a bacterial suspension of *Pst* DC3000 ($5.0*10^8$ cfu/mL) to analyze dual phosphorylation of the TEY motif by SDS-PAGE, western blotting (WB), and immunodetection with polyclonal antibodies. Prior to immunodetection, the blot was stained with Ponceau S to assess whether loading was equal. The experiment was done three times with similar results. Note the absence of pTEpY immunodetection bands in the *mpk3* and *mpk6* mutant, confirming that the immuno-reactive material is phosphorylated MPK3 and MPK6.

Priming Is Accompanied by Activation of Various Genes Encoding Different Components of MAPK Cascades

The results shown in Figure 9 strongly suggest that MPK3 and MPK6 play a major role in BTH-induced priming for the enhanced defense response against both biotic and abiotic stress. To elucidate whether other MAPK signaling components might potentially support the augmented defense response in primed plants, the publicly available microarray data of BTH-treated wild-type and *npr1* plants (Wang et al., 2006) was further analyzed. In wild-type *Arabidopsis* plants, only 2 *MPK* (*MPK3* and *MPK11*) and 4 *MKK* (*MKK1*, *MKK2*, *MKK4* and *MKK5*) transcripts were induced ≥ 2-fold over the untreated control shortly after priming with 60µM BTH (Fig. 11). Remarkably, comparison of the datasets of Col-0 plants with the *npr1* mutant data revealed that the induction of these 6 MAPK cascade signaling components depends on a functional *NPR1* gene (Fig. 11).
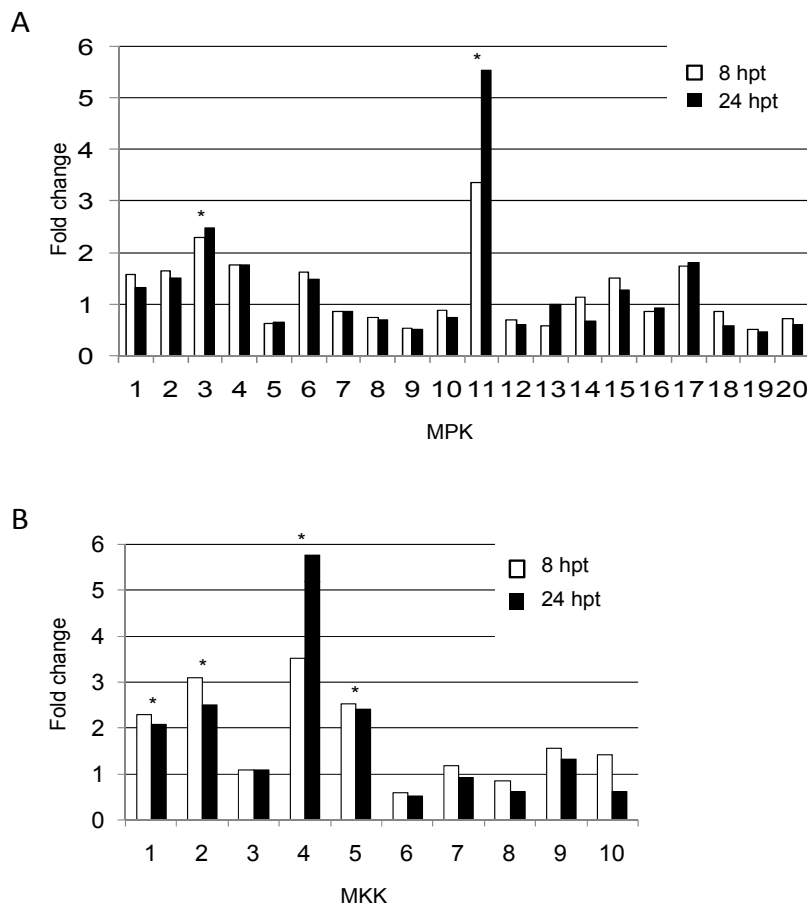
Figure 11: Multiple genes encoding MAPK signaling components are activated upon BTH-induced priming of wild-type (Col-0), but not *npr1* plants.
Relative expression values were extracted from available microarray expression data published online by (Wang et al., 2006). Fold change ratios (t=8h/t=0h or t=24h/t=0h) are based on transcript profiles of Col-0 and *npr1* plants at 0, 8 and 24h after BTH (60µM) application.
(A) Fold-change ratios of the 20 known Arabidopsis *MPK* genes.
(B) Fold-change ratios of 10 known Arabidopsis *MKK* genes. Asterisks indicate transcript induction ≥ 2-fold.

The *MKK1* Gene Is Induced and MKK1 Protein Accumulates to High Levels in BTH-Primed *Arabidopsis* Plants

In a microarray experiment aimed to identify key players in the SAR response of plants, out of the above mentioned MAPK components only the SA-inducible *MKK1* gene was found to be regulated by NPR1 directly (Wang et al., 2005; Wang et al., 2006). After verification of the activation of *MKK1* during priming with BTH (Fig. 12), it was shown that the upregulated transcript level results in MKK1 protein accumulation in wild-type Col-0 plants (Fig. 12). The upregulation of *MKK1* transcripts and protein however, was absent in *npr1* (Fig. 12). Thus, induction of the primed state in *Arabidopsis* Col-0 plants correlates with the upregulation of several components of the MAPK-signaling network that do not accumulate in the priming mutant *npr1.*

Validation of a Differential Phosphoproteomics Approach to Identify Putative Regulators of Priming

The results shown in Figure 10 strongly suggest that priming involves the upregulation of a broad spectrum of latent signaling components, among which MPK3 and MPK6 were first identified as critical regulators of the primed state (Fig. 6). In order to identify further phosphoproteins that might play a role in the regulation
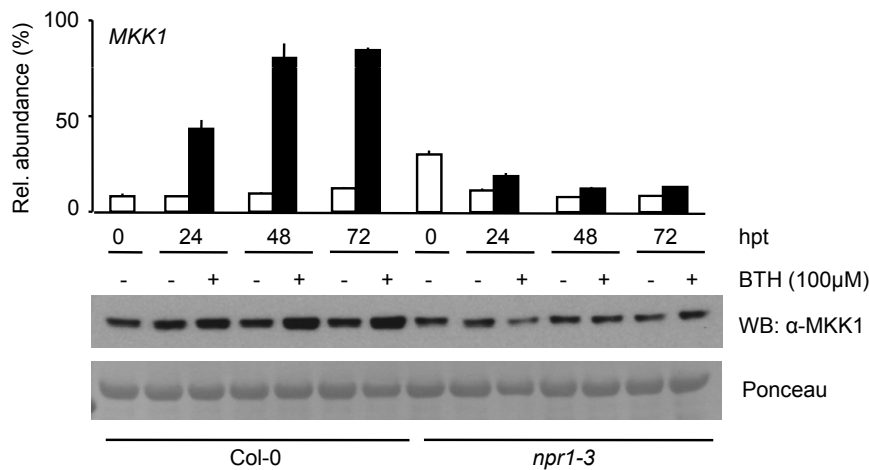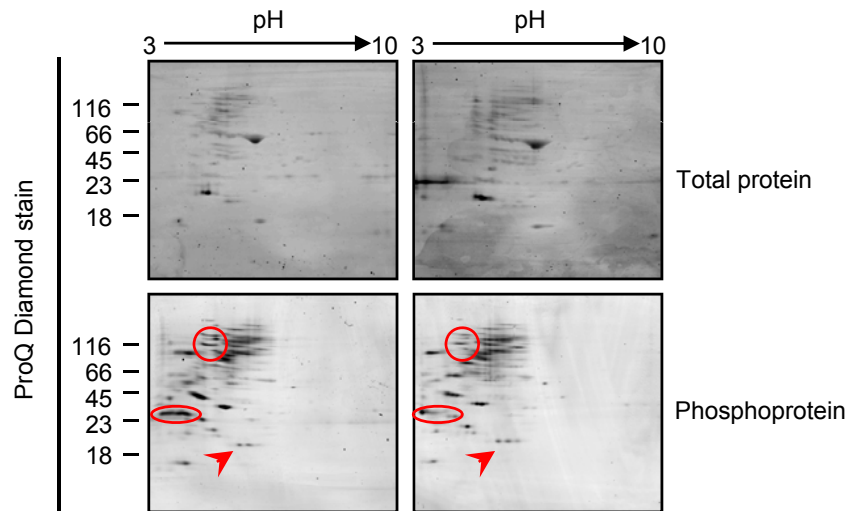
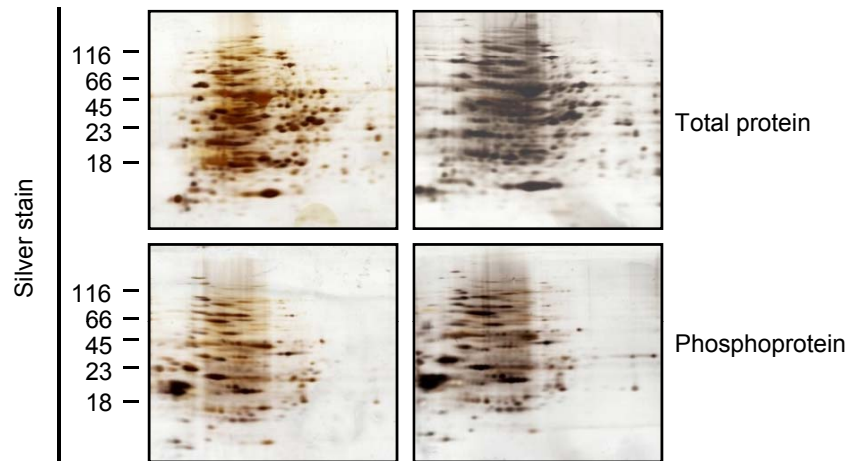**Figure 12: The BTH-inducible MKK1 gene is not upregulated in *npr1*.**
Leaves were harvested at various times after treatment of wild-type and mutant *npr1* plants with 100μM BTH (+) or a wettable powder carrier control (-). An aliquot of leaf tissue was used for RNA extraction and RT-qPCR analysis to examine the relative abundance of transcripts for *MKK1* normalized to those of *ACTIN2*. Another aliquot of leaves was used for protein extraction and SDS-PAGE followed by immunodetection of MPK3 with polyclonal antibodies. Prior to immunodetection, the blot was stained with Ponceau S for equal loading. The experiments were performed three times with similar results. Hpt, hours post treatment; WB, western blot.

of priming for the enhanced stress response, a proteomics approach was conducted by comparison of differentially phosphorylated phosphoproteomes derived from BTH-primed and non-primed plants both challenged by water infiltration. To that end, plants were primed by foliar application of 100μM BTH or wettable powder solution. Three days later, plants were infiltrated on their leaves with tap water. Infiltrated leaves were harvested 10 min after infiltration and total protein was obtained from leaves by phenol-extraction (Weckwerth et al., 2004). Subsequently, phosphoproteins were enriched from 5mg of total protein by metal oxide affinity chromatography (MOAC) that is based on the affinity of the phosphate group for aluminium hydroxide ($Al(OH)_3$) (Wolschin et al., 2005). To maximize sensitivity and to increase the probability that the enriched protein spots indeed represent phosphoproteins, these were specifically stained using the phosphoprotein-specific dye ProQ® Diamond. Comparison of the gels stained with ProQ® Diamond and silver nitrate revealed that the phosphoproteins are not equally spread across the entire pH range of 3-10. Rather, more protein spots are located in the acidic region (Fig. 13A, B). The observation of horizontal streaking of the fluorescent signal was caused by resolving several different peptides with similar molecular mass and isoelectric point (pI). Comparison of the phosphoproteomes of BTH-primed and non-primed plants, both challenged by water infiltration, revealed several dramatic changes in the phosphorylation state of various protein spots that became visible upon ProQ® Diamond staining (Fig. 13A). Furthermore, some spots show a "string of pearls" appearance (Fig. 13A) which is typical for proteins with multiple phosphorylation sites (Wolschin et al., 2005). In several cases, the intensities of the individual spots of such strings of pearls vary

A

ProQ Diamond stain

pH 3 → 10   pH 3 → 10

116
66
45
23
18

Total protein

116
66
45
23
18

Phosphoprotein

B

Silver stain

116
66
45
23
18

Total protein

116
66
45
23
18

Phosphoprotein

C

116
66
45
23
18

← MPK 3

WB: α-MPK3

D

116
66
45
23
18

← T*Y
← MPK 6
← MPK 3

WB: α-pTEpY

−   +   100µM BTH, 72 hr
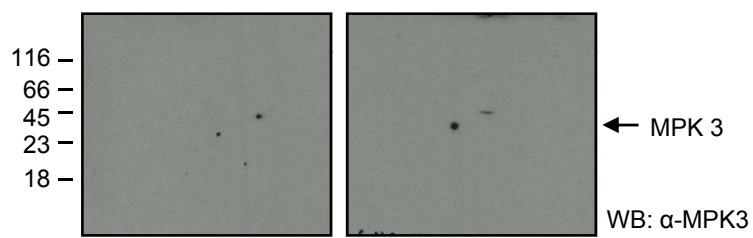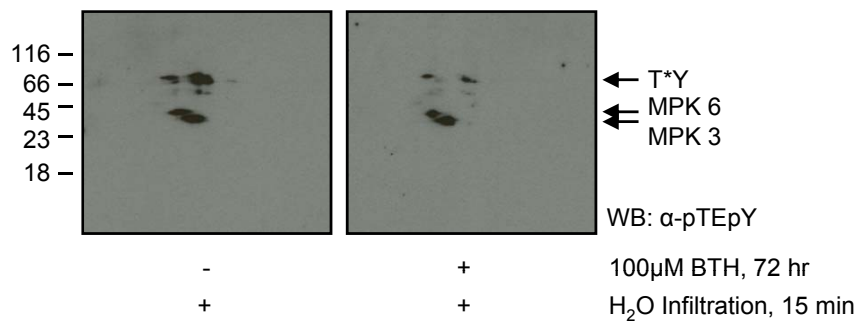+   +   H₂O Infiltration, 15 min

for legend, see next page

Figure 13: Comparison of MOAC-enriched phosphoprotein samples proofs to be a powerful proteomics approach to identify novel regulators of priming.
Col-0 wild-type plants were primed by spraying 100µM BTH (+) or treated with wettable powder solution (-). After three days, plants were challenge-infiltrated with tap water. Challenged leaf tissue was harvested and frozen in liquid nitrogen 10 minutes after challenge treatment. From ~4g of ground leaf material, total protein was extracted. Subsequently phosphoprotein was enriched using 5mg resolubilized total protein. Both total protein and phosphoprotein-enriched fractions were loaded on isoelectric focussing gels. Separation in the first dimension was performed by isoelectric focussing over the pH range 3-10 and in the second dimension on NuPAGE 4-12% Bis-Tris gels.
(A) Gels were incubated in ProQ® Diamond fluorescent gel stain solution to detect phosphoproteins. Red arrowheads indicate shifts of the intensity of individual phospho-isoforms within a "string of pearls".
(B) Subsequently total protein was stained by incubation of the gels in silver nitrate solution.
(C) Aliquots of MOAC-enriched phosphoprotein fractions were used for western blot analysis using MPK3-specific polyclonal antibodies.
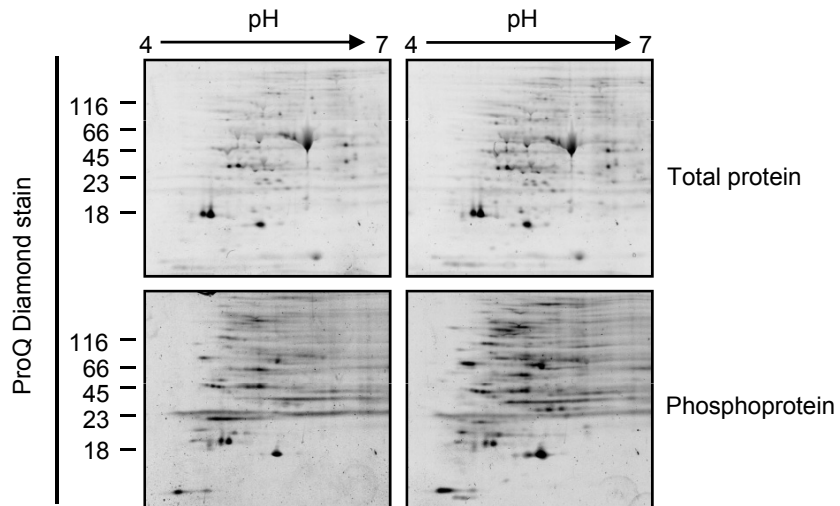**(D)** Same experimental setup as in (C) but detecting phosphorylation of the TEY motif using polyclonal antibodies.

among the different samples indicating a shift of protein phosphorylation(s) upon challenge treatment of primed vs. non-primed plants (Fig. 13A). To validate whether this phosphoproteomics approach is applicable to identify novel regulatory phosphoproteins involved in the molecular mechanism of priming, the abundance of MPK3 proteins in the phosphoprotein enriched samples was examined as a positive control. The immunoblots in Figure 13C show that the MOAC protocol enriched more phosphorylated MPK3 proteins from BTH-primed plants vs. non-primed plants after infiltration of water into leaves. Strikingly, immunodetection of proteins that possess a dually phosphorylated pTXpY motif indicates that several proteins with an apparent molecular mass between 45 and 116 kDa are likely to be augmentedly de-phosphorylated upon water infiltration of primed leaf tissue (Fig. 13D). Protein sequence BLAST searches of the *Arabidopsis* annotated proteome elucidated that these spots likely correspond to MPK proteins belonging to the clade of MPKs (Fig. 13D), whose members all display a distinctive TDY, rather than TEY signature (Hamel et al., 2006).

Identification of Novel Regulators of Priming

After validation of the proteomics approach described above, with MPK3 as a positive control, the MOAC protocol was now exploited to identify novel candidate regulators for the primed state in *Arabidopsis*. Again, BTH-primed and non-primed *Arabidopsis* plants were challenged by water infiltration and total protein and phosphoprotein-enriched samples were prepared from infiltrated leaf tissue. To optimize the resolution of the protein spots visualized within gels and, thus, to enable easy spot picking by a robot, samples were loaded onto immobilized pH gradient (IPG) strips ranging from pH 4 to 7 for first dimension isoelectric focusing (IEF)(Fig. 14). Many differentially phosphorylated protein spots became visible upon staining of phosphoproteins with ProQ® Diamond (Fig. 14A). All protein spots picked were in-gel trypsin digested and the resulting peptides analyzed by MS. The identified protein spots are listed in Table 1 and sorted by molecular weight. After spot picking, the gels were incubated in colloidal PageBlue™ solution

A

ProQ Diamond stain

pH 4 → 7   pH 4 → 7

116
66
45
23
18

Total protein

116
66
45
23
18

Phosphoprotein

B

PageBlue™ Coomassie stain

116
66
45
23
18

Total protein

116
66
45
23
18

Phosphoprotein

C

116
66
45
23
18

← MPK 3

WB: α-MPK3

D

116
66
45
23
18

← T*Y

← MPK 6
← MPK 3

WB: α-pTEpY

−   +   100μM BTH, 72 hr
+   +   H₂O Infiltration, 15 min
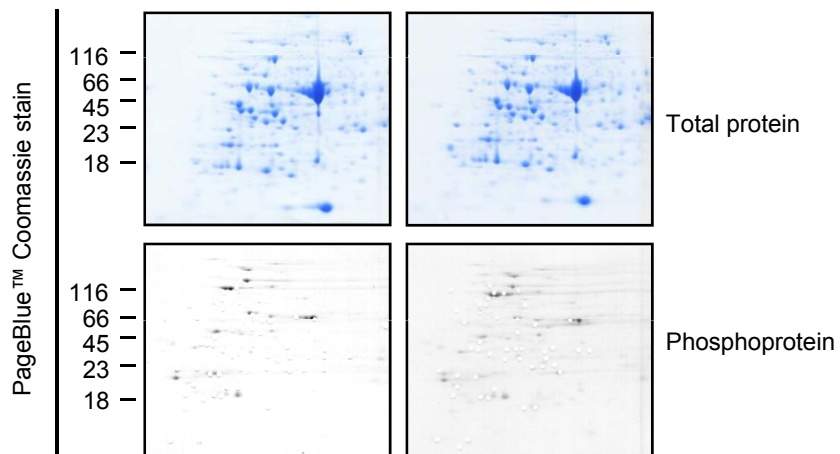
Figure 14: High resolution separation of protein spots via 2D PAGE of primed vs. non-primed tissues both challenged by water infiltration.

Plants were either pretreated with BTH (100µM) or wettable powder solution for 3 days. Leaves were then infiltrated with water and used to extract total protein fractions and enrich phosphoproteins. Concentration of total protein was determined by Bradford assay and equal protein amounts were loaded on IPG strips (pH 4-7). Second dimension was performed using NuPAGE 4-12% Bis-Tris gels. Equal volumes of phosphoprotein-enriched fractions were separated as described in the legend to Fig. 13.

(A) ProQ® Diamond-stained phosphoprotein.
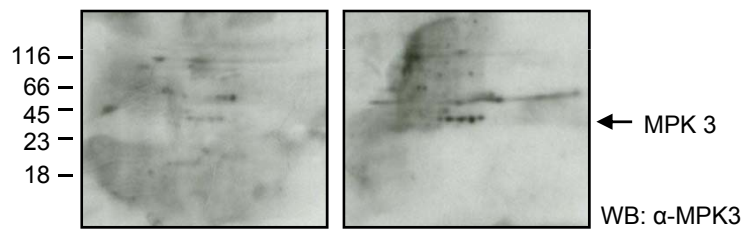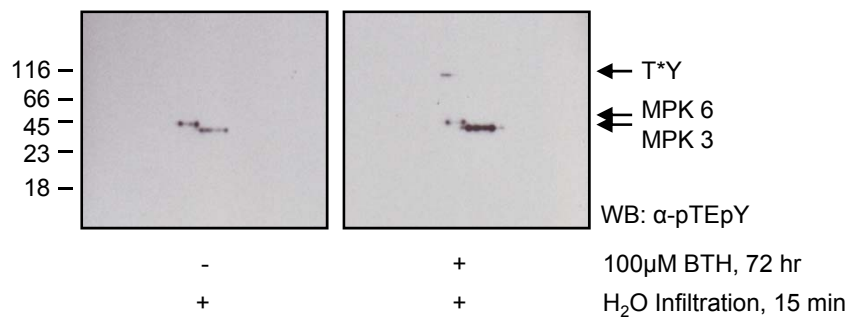**(B)** PageBlue™ staining of total protein.
(C) Aliquots of phosphoprotein-enriched samples were used for immunoblot analyses using MPK3-specific polyclonal antibodies.
(D) Same experimental setup as in (C) but assaying phosphorylation of the TEY motif using pTEpY-specific antibodies.

to stain total proteins and ensure equal loading (Fig. 14B). As an internal positive control, phosphorylated MPK3 protein levels were assayed using MPK3-specific polyclonal antibodies on immunoblots prepared from aliquots of the phosphoprotein enriched fractions (Fig. 14C). Interestingly, the immunoblots that were incubated with pTEpY-specific antibodies recognized at least 4, and possibly 6 isoforms of the MPK3 protein whereas only 2 isoforms were detected for MPK6 (Fig. 14D). Furthermore, a faint protein band of high molecular mass cross-reacted with the antibodies and only appeared in the phosphoprotein-enriched sample that was prepared from primed and subsequently challenged plants (Fig. 14D). Further evidence for the specificity of ProQ® Diamond was gathered when duplicate 2D blots were incubated in the presence or absence of λ protein phosphatase. On-blot dephosphorylation of phosphoproteins confirmed the presence of phosphate groups covalently linked to the protein spots detected by subsequent ProQ® Diamond blot staining (Fig. 15). Due to dephosphorylation of the phosphoproteins, the intensity of ProQ® Diamond staining of phosphoproteins after treatment with λ-phosphatase decreased (Fig. 15).
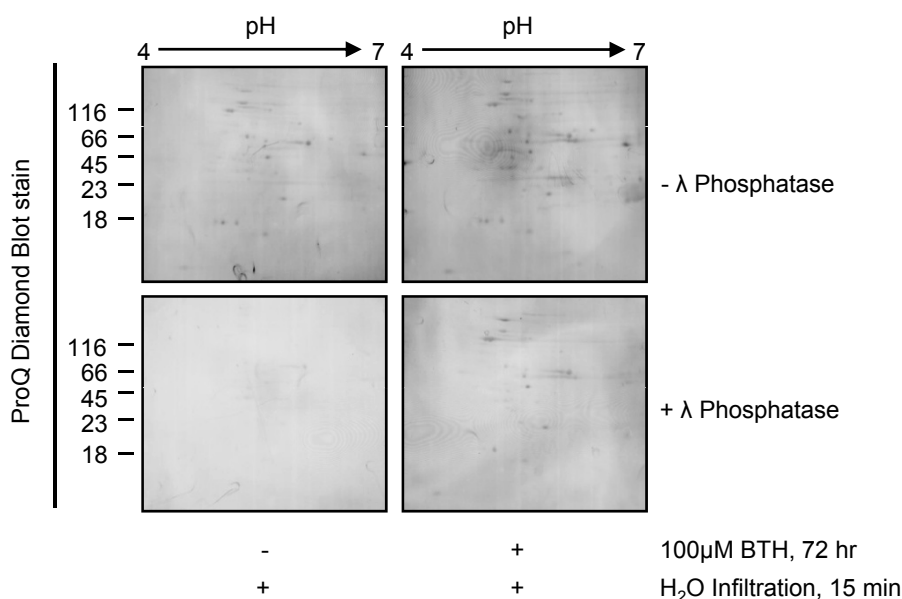
for legend, see next page

Figure 15: On-blot dephosphorylation of phosphoproteins confirms the specificity of ProQ® Diamond detection of phosphoproteins.
Duplicate immunoblots were prepared from phosphoprotein-enriched samples as described in the legend to Fig. 14. Images were acquired after immersing the blots in ProQ® Diamond blot staining solution.

Table 1: List of phosphoproteins from challenge-infiltrated, BTH-primed and non-primed *Arabidopsis* plants.
Experiment was performed as described in the legend to Fig. 14. Upon phosphoprotein staining, protein spots were picked with a robot and subsequently transferred to 96-wells plates for automated in-gel tryptic digestion and target preparation. PMFs were acquired with MALDI-MS and MS/MS analyses and used for database searches with the Mascot 2.2 software.

GenInfo	Protein	Predicted MW (kDa)	Predicted pI
gi|23397152	putative heat shock protein 81-2 (HSP81-2)	80	4.8
gi|15241844	luminal binding protein 1 (BiP-1) (BP1)	73.6	4.9
gi|1695717	luminal binding protein	73.6	4.9
gi|30693962	BIP (LUMINAL BINDING PROTEIN); ATP binding	73.5	5
gi|42572263	ACBP4 (ACYL-COA BINDING PROTEIN 4)	73.2	5
gi|15241847	heat shock cognate 70 kDa protein 2 (HSC70-2) (HSP70-2)	71.3	4.9
gi|15241849	HSC70-1 (heat shock cognate 70 kDa protein 1); ATP binding	71.3	4.9
gi|397482	heat shock protein 70 cognate	71.3	4.9
gi|425194	heat shock protein	70.8	5
gi|18420426	ubiquitin-specific protease 8, putative (UBP8)	70.2	5.4
gi|15240485	ATMAP65-1 (MICROTUBULE-ASSOCIATED PROTEINS 65-1)	65.7	4.8
gi|15982735	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	60.4	5.1
gi|4539465	putative protein [Arabidopsis thaliana]	56.5	4.5
gi|7525018	ATP synthase CF1 alpha subunit	55.3	5
gi|42565672	PTAC16 (PLASTID TRANSCRIPTIONALLY ACTIVE18)	54.3	9.4
gi|15222929	(VACUOLAR ATP SYNTHASE SUBUNIT B1)	54.1	4.8
gi|7525040	ATP synthase CF1 beta subunit	53.9	5.3
gi|904147	adenosine triphosphatase	53.9	5
gi|7525041	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	52.9	5.9
gi|18405145	RCA (RUBISCO ACTIVASE)	51.9	5.8
gi|131979	RBL_HUMBA Ribulose bisphosphate carboxylase large chain	51.5	6.2
gi|15241839	unknown protein [Arabidopsis thaliana]	48.2	4.8
gi|15227987	LOS2 (Low expression of osmotically responsive genes 1)	47.7	5.5
gi|18175728	putative enolase (2-phospho-D-glycerate hydroylase)	47.7	5.5
gi|15227978	lipase, putative	46	5
gi|15237869	VHS domain-containing protein / GAT domain-containing protein	45.3	4.6
gi|15232408	D-fructose-1,6-bisphosphate 1-phosphohydrolase, putative / FBPase	45.1	5.1
gi|15222551	PRK (PHOSPHORIBULOKINASE); ATP binding / phosphoribulokinase	44.4	5.7
gi|15231039	ATG2 (G2p-related protein); metalloexopeptidase	43	6.4
gi|18420348	fructose-bisphosphate aldolase, putative	43	7.5
gi|13430628	AF360226_1 putative nuclear DNA-binding protein G2p	42.9	6.4
gi|14334740	putative fructose bisphosphate aldolase	42.9	6.6
gi|18399660	fructose-bisphosphate aldolase, putative	42.9	6.2
gi|15228194	SBPASE (sedoheptulose-bisphosphatase); phosphoric ester hydrolase	42.4	6.2

gi|21592624	hydroxymethylbilane synthase	41.1	9.7
gi|15239282	ATLFNR1 (LEAF FNR 1); NADPH dehydrogenase/ oxidoreductase	40.3	9.1
gi|18414476	unknown protein [Arabidopsis thaliana]	37.2	4.8
gi|15231817	CP29 (chloroplast 29 kDa ribonucleoprotein); RNA binding	36	5.1
gi|21592483	similar to late embryogenesis abundant proteins	35.9	4.5
gi|4204267	Unknown protein [Arabidopsis thaliana]	35.3	9.3
gi|18420800	30S ribosomal protein, putative	34.8	6.3
gi|6690272	AF114386_1 putative protease HhoA precursor	34.6	5
gi|681904	RNA-binding protein cp29	34.5	5.1
gi|15232603	60S acidic ribosomal protein P0 (RPP0B)	34.1	4.8
gi|15237716	KH domain-containing protein NOVA, putative	33.8	5.6
gi|51969936	unnamed protein product [Arabidopsis thaliana]	33.5	5.4
gi|30686081	VHS domain-containing protein / GAT domain-containing protein	33.3	4.7
gi|15242123	band 7 family protein [Arabidopsis thaliana]	32.4	5.2
gi|4741948	AF134124_1 Lhcb2 protein	28.6	5.4
gi|18416966	universal stress protein (USP) family protein	28.1	5.8
gi|51970852	unknown protein [Arabidopsis thaliana]	28.1	5.8
gi|18403546	LHB1B2 (Photosystem II light harvesting complex gene 1.5)	28	5.2
gi|51971835	unknown protein [Arabidopsis thaliana]	28	5.9
gi|25299531	E86426 probable elongation factor 1-beta [imported]	26.5	4.4
gi|30691619	elongation factor 1-beta / EF-1-beta	25.1	4.3
gi|16374	chlorophyll a/b binding protein (LHCP AB 180)	25	5
gi|9295717	AC006535_1 T24P13.1 [Arabidopsis thaliana]	23.8	6.6
gi|12325357	AC024261_3 RNA-binding protein, putative; 35994-37391	21	4.6
gi|15240912	ribulose bisphosphate carboxylase small chain 1B (RBCS-1B) (ATS1B)	20.3	8.9
gi|132091	RBS2_BRANA Ribulose bisphosphate carboxylase small chain F1	20.2	9.2
gi|15222741	eukaryotic translation initiation factor 5A, putative / eIF-5A, putative	17.1	5.5

DISCUSSION

Priming of cells for more efficient activation of defense reactions to biotic and abiotic stress has been associated with the acquired immunity response of many organisms (Conrath et al., 2002; Conrath et al., 2006). Although priming phenomena are increasingly well-studied in both animals and plants, the molecular mechanisms underlying priming are not yet understood. It was previously hypothesized that the primed state is the result of the accumulation of latent cellular signaling components that, upon subsequent perception of stress, become "hyperactivated" by a second signaling event, triggering an augmented signal transduction (Conrath et al., 2006). Until now, the identity of these hypothetical signaling components remained obscure. Here, first functional evidence is provided for a molecular mechanism that mediates priming of *Arabidopsis thaliana* for more rapid and stronger activation of defense against stress. It was demonstrated that pre-stress deposition of two members of the MAP kinase family of signaling enzymes, MPK3 and MPK6, play a key role in priming in *Arabidopsis*. MPKs are known to be important components of eukaryotic signal transduction and transmute extracellular stimuli into intracellular responses, thereby amplifying signal transduction (Hamel et al., 2006). Thus, MPKs are prime candidates for cellular signaling enzymes that could mediate priming.

Numerous molecular and biochemical studies have revealed that in *Arabidopsis* MPK3/MPK6 play an important role in the response to a broad variety of biotic and abiotic stresses, including wounding, pathogen infection, temperature, ozone, drought and salinity, but also in the signaling of plant hormones, ethylene, methyl jasmonate (MeJA), abscisic acid and SA (Mizoguchi et al., 1996; Nakagami et al., 2005). Whereas the enzyme activity of both MPK3 and MPK6 seems to be induced in response to many types of treatments, transcriptional regulation of *MPK3/MPK6* is less well understood. Previous microarray studies revealed induction of *MPK3* by MeJA and SA (Schenk et al., 2000). In addition, transcripts of *MPK3* also increased in *Alternaria brassicicola*-infected *Arabidopsis* leaf tissue as well as in the distal non-infected leaves 72 hours after inoculation (Schenk et al., 2000; Schenk et al., 2003). Here, it is shown that *MPK3* and *MPK6* transcripts and proteins are upregulated upon induction of the primed state in *Arabidopsis* by treatment with a synthetic inducer of acquired resistance, BTH (Fig. 2). Furthermore, when priming was induced in wild-type *Arabidopsis* by inoculation of three lower leaves with avirulent bacteria, MPK3 and MPK6 proteins accumulated in infected and systemic leaves (Fig. 3, 10). Although induction of the primed state strongly correlated with the induction of both proteins, the enzymes accumulated in their non-phosphorylated state, and thus remained inactive in primed *Arabidopsis* (Fig. 2, 3, 6). However, upon a secondary challenge treatment, MPK3/MPK6 proteins became more rapidly and strongly phosphorylated in primed plants resulting in an augmented MPK activity when compared to the non-primed control plants (Fig. 4). The observation of enhanced MPK activity in BTH-primed wild type plants that were subsequently stressed, strongly correlated with a better activation

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of the defense marker genes *PAL1*, *PR1*, *4CL* and *C4H* (Fig. 6). In the two *mpk3* mutants, priming by BTH and the resulting potentiation of infiltration-induced defense gene expression was absent or largely alleviated (Fig. 6). By contrast, in *MPK6*-silenced plants and the *mpk6* mutant, the priming-mediated enhancement of infiltration-induced defense gene activation was only moderately affected (Fig. 6). Therefore, it is likely that MPK3 is a major component in the BTH-induced priming for potentiated defense gene activation in *Arabidopsis*, while MPK6 presumably serves a more minor role (Fig. 6). This conclusion is supported by (*i*) the stronger accumulation of MPK3 in BTH-primed plants when compared to MPK6 (Fig. 2, 4A), (*ii*) the more intense signal of the phosphorylation of the TEY activation motif in MPK3 (Fig. 4A), and (*iii*) the higher increase in MPK3's enzyme activity (Fig. 4B).

The SAR-deficiency mutant *npr1* has already been shown to be unable defective in priming for *PAL1* gene expression upon water infiltration of BTH-pretreated leaves (Kohler et al., 2002). Here, it is shown that MPK3/MPK6 proteins do not accumulate in *npr1* mutant plants upon BTH treatment (Fig. 7A), resulting in a dramatically lowered infiltration-induced phosphorylation of the TEY motif of MPK3/MPK6 (Fig. 7B) and a loss of acquired immunity (Cao et al., 1994). The finding that *MKK1* is a direct target of NPR1 (Fig. 11, 12) and that MKK1 proteins do not accumulate in the *npr1* mutant upon BTH treatment (Fig. 12) suggests that AI might be regulated at different levels of the hierarchical MAPK signaling cascade. Although data of several groups demonstrated that MKK1 is unable to physically interact with, and activate MPK3/MPK6 in *Arabidopsis* (Asai et al., 2002; Matsuoka et al., 2002; Teige et al., 2004; Meszaros et al., 2006), it is tempting to speculate that the reduced levels of MKK1 and MPK3/MPK6 in BTH-treated *npr1* (Fig. 7A, 12) contributes to the loss of acquired immunity in these plants. Accordingly, constitutively primed *edr1* plants display enhanced stress resistance and possess higher basal levels of MPK3 when compared to the untreated wild type plants (Fig. 8). A challenge treatment by water infiltration of the *edr1* mutant induced enhanced TEY phosphorylation in both primed and non-primed plants (Fig. 8). Infiltration-induced TEY phosphorylation was stronger in *edr1* compared to the wild type which was associated with a higher induction of *PAL1* transcripts (Fig. 8). Although it cannot be excluded that the genetic defects in these disease resistance mutants mediate modifications in upstream signal transduction components that might cause modified phosphorylation of MPK3/MPK6, the *edr1* data (Fig. 8) strongly support the proposed key role for MPK3 in priming of *Arabidopsis*. Several different lines of evidence further corroborate that priming is mediated mainly by MPK3. Firstly, the close correlation between the ability of compounds to induce priming (Kohler et al., 2002) and their capability to activate the *MPK3* gene in *Arabidopsis* (Fig. 1B). Secondly, the greater decrease in BTH-induced immunity in *mpk3* when compared to *mpk6* (Fig. 8). Moreover, an earlier report by Menke et al. (2004) demonstrated that *MPK6*-silenced *Arabidopsis* plants, in contrast to *mpk3*, were not significantly

affected in their ability to develop AI. However, as priming for enhanced *PAL1* and *PR1* expression was somewhat affected in *MPK6* RNAi plants and/or the *mpk6* mutant (Fig. 6), both MPK3 and MPK6 seem to be important for complete priming in *Arabidopsis*. This may explain why knockout of only one of the two genes encoding MPK3 or MPK6 doesn't always completely abolish SAR (Fig. 8, 9). Indeed, physiological interaction (Miles et al., 2005) and substrate overlap (Feilner et al., 2005) have been shown for MPK3 and MPK6, and it is known also that the tobacco orthologs of *Arabidopsis* MPK3 and MPK6, wound-induced MAP kinase (WIPK) and salicylic acid-induced MAP kinase (SIPK), interact with each other *in planta* (Liu et al., 2003). The finding that hyperactivation of MPK6 in the infiltrated as well as primed and afterwards infiltrated *mpk3* mutant does not correlate with further enhanced potentiation of *PAL1* or *PR1* expression (Fig. 6) also argues against a commanding role of MPK6 in priming. An additional experiment to support this hypothesis would be the constitutive expression of *MPK3* in *Arabidopsis*. However, various efforts to overexpress the *MPK3* gene in *Arabidopsis* under control of constitutive and inducible promoters were not successful (data not shown). In fact, stably transformed transgenic plants expressing *MPK3* under control of the 35S Cauliflower Mosaic Virus promoter exhibited 2 to 3-fold increased *MPK3* transcript levels yet these plants did not possess enhanced basal levels of MPK3 protein and lacked a constitutively primed phenotype (data not shown). However, constitutive expression of *MPK3* orthologs at high levels in other plant species was demonstrated to enhance their resistance to pathogens. For example, overexpression of the Trichoderma-Induced Protein Kinase (*TIPK*) in cucumber led to constitutively enhanced resistance to the leaf pathogen *P.s.* pv. *lachrymans* (Shoresh et al., 2006). Moreover, in rice transgenic overexpression of *MK1*, the pepper homolog of *MPK3*, conferred resistance to rice blast disease (Lee et al., 2004).

Systemic enrichment of MPK3 proteins was observed when SAR was induced in wild-type *Arabidopsis* by inoculation of three lower leaves with *Pst* or *Psp* carrying either the avirulence gene *avrRpt2* or *avrB* respectively (Fig. 3). The systemic MPK3 accumulation was associated with enhanced resistance against challenge inoculation of wild-type plants with *Pst* DC3000 (Fig. 10A) (Kohler et al., 2002). The latter, however, could not be observed in similarly infected *mpk3* mutants (Fig. 10A). The slightly reduced level of BTH-induced immunity in both *mpk3* and *mpk6* mutants (Fig. 9) is ascribed to the fact that both, MPK3 and MPK6 contribute to AI in *Arabidopsis* and to functional redundancy of MPK3 and MPK6 (Wang et al., 2007). In fact, the finding that MPK6 activity is induced to extraordinarily high levels in leaves of the infiltrated as well as primed and afterwards infiltrated *mpk3* mutant (Fig. 6B) suggests that the plant tries to compensate lack of MPK3 activity by hyperactivation of MPK6. Recently, it was suggested that a mechanism would exist that compensates for missing MPK3 by enhancing the activation of MPK6 but not *vice versa* (Wang et al., 2008). Unfortunately, simultaneous knockout of *MPK3* and *MPK6* is embryo-lethal (Wang et al., 2007). However,

to conditionally rescue the embryo lethality of *mpk3/mpk6* double mutants, *MPK6* was introduced under control of a dexamethasone-inducible promoter (Wang et al., 2007). After germination, the $mpk3^{-/-}$ $mpk6^{-/-}$ seedlings are arrested at the cotyledon stage (Wang et al., 2007). They are compromised in production of the phytoalexin camalexin which is required for resistance of *Arabidopsis* against *Botrytis cinerea* (Ferrari et al., 2007; Ren et al., 2008). It will be interesting to see whether the *mpk3/mpk6* double mutant exhibits deficiency in developing AI.

A recent report demonstrated that HopAI1, a bacterial effector protein that contributes to *Pst* virulence in plants, acts as a phosphothreonine lyase, which removes the phosphate group from phosphothreonine to permanently inactivate MPKs (Li et al., 2007). HopAI1 directly interacts with *Arabidopsis* MPK3 and MPK6 (Zhang et al., 2007). In *Arabidopsis* HopAI1 also suppresses endogenous MPK3 and MPK6 activation by bacterial flagellin and dampens the associated immune response (Zhang et al., 2007). Thus, upregulation of MPK3 and MPK6 levels seems to enhance, and downregulation of MPK3/MPK6 activity reduce defense in *Arabidopsis*. Interestingly, the plant pathogen *Agrobacterium* counters the MPK-mediated defense by abusing the plant's own MPK3 to phosphorylate the plant transcription factor VIP1 during delivery of its infectious transfer DNA into the host plant (Djamei et al., 2007).

Also, a strategy is described here for the identification of phosphoproteins with a potentially regulatory role in priming. The method uses purification of denatured proteins by a phenol-based extraction, enrichment of phosphoproteins by MOAC, 2-D PAGE, phosphoprotein-specific staining, and MS analysis. The approach allows large-scale analysis of *in vivo* phosphorylation/dephosphorylation processes of plant proteins that occur in different experimental treatments/situations. The combination of phosphoprotein enrichment by MOAC and phosphoprotein specific staining provides a high level of certainty that spots which became visible by ProQ™ Diamond staining indeed correspond to phosphorylated proteins.

Validation of the methodology by monitoring the abundance of MPK3 as a reference protein (Fig. 13, 14) not only provided insight into the efficiency and selectivity of this strategy, but also increased the probability of newly identified candidate phosphoproteins that are involved in priming. More than 60 proteins were identified by MS analysis. For many of these, published evidence suggests that they exist as phosphoproteins (Cascardo et al., 2000; Lintala et al., 2007). In contrast to several proteins which seem to be constitutively present in the primed and control situation, spot intensities of most phosphoproteins vary upon priming *Arabidopsis*. This may be explained by phosphorylation/dephosphorylation of constitutively expressed proteins and/or priming-induced synthesis or degradation of phosphoproteins. Furthermore, the identification of proteins in more than one spot indicates multiple phosphorylation states of these proteins.

Priming-induced shifts of spot intensities were observed for several phosphoprotein spots indicating that priming involves proteome-wide regulatory phosphorylation/dephosphorylation events.

One interesting protein that has been identified in the above mentioned approach as another phosphoprotein that possibly is involved in establishment of the primed state in *Arabidopsis* is the Ferrodoxin-NADP$^+$ Reductase (FNR1) (Table 1). As FNR1 was more abundant in primed than in non-primed control plants this suggests that infiltration-induced phosphorylation of FNR1 is enhanced in primed plants. Interestingly, FNR of spinach was activated *in vitro* upon covalent binding of ATP (Hodges and Miginiac-Maslow, 1993) and FNR overexpression in tobacco increases the tolerance to oxidative stress (Rodriguez et al., 2007). Therefore it is tempting to speculate that FNR accumulates in primed *Arabidopsis* and becomes hyperactivated upon challenge treatment, thereby suppressing the so-called "oxidative burst" in primed plants. Another interesting candidate that was identified as a potential mediator of priming in *Arabidopsis* with the above mentioned technology, is the luminal binding protein 1 (BiP1), an ER-resident molecular chaperone (Koizumi, 1996). Recently, BiP proteins were demonstrated to be involved in SAR of *Arabidopsis* by facilitating folding and secretion of defense-related proteins that are targeted to the apoplast (Wang et al., 2005). The experimental layout of the proteome-wide screen for novel regulatory phosphoproteins of priming includes the advantageous possibility that the identified proteins are direct or indirect phosphorylation substrates of MPK3/MPK6. In the future, further MOAC-enriched phosphoprotein fractions need to be analyzed to determine the time course of phosphorylation events upon infiltration of water into *Arabidopsis* leaves. Furthermore, premium candidate proteins need to be studied in more detail to address their exact biological function in the establishment of the primed state in *Arabidopsis*.

Over the past few years, priming-inducing compounds were shown to be potent inducers of stress resistance also in crop plants (Beckers and Conrath, 2007). Moreover, some modern agronomic plant-protecting compounds such as the insecticide Imidacloprid and the strobilurin fungicide Pyraclostrobin have been shown to possess priming-inducing activity in the greenhouse and field, resulting in improved yield (Bartlett et al., 2002; Thielert, 2006). For effective and sustainable crop and biomass protection in the future, a better understanding of the molecular mechanisms underlying priming of plant defense responses as well as the mapping and characterization of additional molecular markers of priming will provide new possibilities for the development of novel plant-protecting strategies. These not only include modern pesticides that combine anti-microbial and priming-inducing activity, but might also comprehend transgenic plant-protecting approaches.

Priming is a phenomenon that has been associated with different types of induced resistance (Con-

rath et al., 2006). Priming boosts inducible defense responses that are activated in a host upon pathogen attack. Priming phenomena have not only been described in plants. For example, in *Drosophila melanogaster*, priming by sublethal doses of *Streptococcus pneumoniae* protects against an otherwise-lethal second challenge of *S. pneumoniae* (Pham et al., 2007). Moreover, in mammals priming of monocytes with interferon-γ boosts the induction of cytokine secretion subsequently induced by bacterial lipopolysaccharides (Hayes et al., 1995). However, so far very little is known about the molecular mechanisms behind priming in these animals. The finding that lethal factor, a major virulence factor secreted by anthrax bacteria, cleaves MPKs and leads to ineffective priming of T cells (Agrawal and Pulendran, 2004) suggests that MPKs might have a role in priming in mammals as well. It will be interesting to see whether pre-stress deposition of inactive proteins corresponding to *Arabidopsis* MPK3 and MPK6 plays a role in animal priming.

MATERIALS AND METHODS

Growth and Treatment of *Arabidopsis* Plants

Arabidopsis thaliana seeds of wild-type Col-0, *npr1-3*, *edr1-1*, *mpk3* (SALK_151594 and *Δmpk3*) and *mpk6* (SALK_073907 and MPK6 RNAi-silenced) plants were sown in freshly moistened soil (Einheits Erde®, Type VM), stratified at 4° C for 48h before transfer to a growth room at 8h light/16h dark, 20° C, 70% relative humidity. 5-week-old plants were sprayed with a formulation of BTH (100µM) or the wettable powder control, a formulation of BTH void of active ingredient. 3 d later, leaves were left untreated, pressure infiltrated with either tap water or 1µM Flg22 (dissolved in tap water) or dipped into a suspension of phytopathogenic bacteria as described below. Leaf tissue was harvested at the time points indicated in the figure legends.

Pathogen Inoculations and SAR Bioassay

Glycerol stocks of *Pseudomonas syringae* (*Ps*) bacteria were streaked on King's medium B (KB) agar plates containing appropriate antibiotics. Plates were incubated at 28° C for 2 d. A single colony was transferred to liquid KB medium in the absence of antibiotics and grown overnight (o/n) at 28° C in a shaking incubator at 220rpm. Cells were collected by centrifugation at 2,000g at room temperature (RT) and washed twice in 10mM MgCl$_2$. To induce bona-fide SAR, three lower leaves of 5-week-old plants were pressure infiltrated with 5.0*10^6 colony forming units (cfu) of *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000 expressing the *avrRpt2* avirulence gene (Rifampicin 50µg/mL (Rif[50]), Kanamycin 25µg/mL (Kan[25])) (Whalen et al., 1991) or *Pseudomonas syringae* pv. *phaseolicola* (*Psp*) expressing the plasmid-borne *avrB* gene (Rif[50], Tetracycline 10µg/mL (Tet[10])) (Dong et al., 1991). Mock treatments were performed by infiltrating 10mM MgCl$_2$. Subsequently all plants were kept under a dome for 3 d until challenge treatment. Challenge inoculation was performed by dipping plants into a suspension of 5*10^8 cfu *Pst* DC3000 (Rif[50]) or *Ps. maculicola* strain ES4326 (Streptomycin 100µg/mL (Strep[100])) in 10mM MgCl$_2$ containing 0.01% (v/v) silwet L-77 (Dong et al., 1991). Mock inoculations were performed by dipping leaves into MgCl$_2$/silwet L-77 in the absence of bacteria. Again, all plants were kept at high humidity after secondary inoculation until bacterial titer and disease symptoms were estimated 3 or 4 d after infection. For bioassays with *Pst* DC3000 and *Psm* ES4326, leaf discs were harvested and homogenized in 10mM MgCl$_2$ using a MagNa Lyser (Roche) and 3mm glass beads**.** Bacterial counts were assayed by plating serial dilutions of bacteria in 10mM MgCl$_2$ onto KB agar plates containing the appropriate antibiotic(s) and incubating the plates at 28° C for 2 d.

RNA Isolation and Real-Time RT-qPCR

Total RNA was isolated from leaf tissue using TRI Reagent (Molecular Research Center). 1µg of total RNA was incubated with 1U DNaseI (Fermentas) in 10µL at 37° C for 15 min. After DNaseI-inactivation at 70° C for

15 min, RNA was reversely transcribed using 200U Revert Aid™ M-MuLV reverse transcriptase (Fermentas) and 2.5μM random nanomer primers in 20μL sample volume. Diluted cDNA was used as template for real-time RT-qPCR reactions run on an ABI PRISM 7000 sequence detector system (Applied Biosystems). Each 10μL reaction mix contained SYBR Green Master Mix (Applied Biosystems) and 0.2μM gene-specific primers (Table 2). Real-time DNA amplification was analyzed using the ABI PRISM 7000 SDS 1.0 software (Applied Biosystems) and the method (Livak and Schmittgen, 2001). Expression of the *ACTIN2* gene was used as an endogenous reference to normalize differences in template amounts.

Table 2 List of gene-specific forward (F) and reverse (R) primers used for real-time RT-qPCR analysis

Primer name	Primers used for quantitative PCR (5'-3')
F QMPK3	TGACGTTTGACCCCAACAGA
R QMPK3	CTGTTCCTCATCCAGAGGCTG
F QMPK6	CCGACAGTGCATCCTTTAGCT
R QMPK6	TGGGCCAATGCGTCTAAAAC
F QPAL1	AACGGAGGAGGAGTGGACG
R QPAL1	CTTTCATTTGCTCCGCTGC
F QPR1	TCGGAGCTACGCAGAACAACT
R QPR1	TCTCGCTAACCCACATGTTCA
F Q4CL	CGGCTGAGCTAGAGGCTTTG
R Q4CL	GGAACTTCACCAGCTGCTT
F QC4H	CACTCGATAGACCACAATGTTG
R QC4H	GGACAATGTTCTTTACATCGTCG
F QACTIN2	GGTAACATTGTGCTCAGTGGTGG
R QACTIN2	GGTGCAACGACCTTAATCTTCAT

Protein Extraction, SDS-PAGE, Western Blotting, and Immunodetection

The extraction of total protein was carried out on ice by resuspending ground frozen leaf tissue in protein extraction buffer (50mM Tris-HCl pH 8.0, 1mM 2-[2-(Bis(carboxymethyl)amino)ethyl-(carboxymethyl)amino] acetic acid (EDTA), 0.1% (v/v) β-mercaptoethanol (β-MEtOH) and 30μg/mL phenylmethylsulfonyl fluoride

(PMSF)). The extract was centrifuged twice at 12,000 g for 30 min at 4° C and the protein concentration of the supernatant was assayed with Quick Start ™ Bradford Dye Reagent (Bio-Rad) and Bovine Serum Albumin (BSA) as a standard. Typically, 10µg of total protein was mixed with 2X sample buffer (125mM Tris-HCl pH 6.8, 50% (v/v) glycerol, 3% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) β-MEtOH and 0.02% (w/v) bromophenol blue), heated at 95° C for 3 min and analysed by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Prestained molecular mass markers (Fermentas) were used to calculate the mass of protein bands. After electrophoresis, proteins were transferred to a nitrocellulose membrane and equal loading was checked by Ponceau S staining of Rubisco. Membranes were subsequently blocked for 1h at RT in 5% (w/v) skimmed milk powder in Tris-buffered saline (TBS; 20mM Tris-HCl pH 7.6, 137mM NaCl) containing 0.1% (v/v) Tween-20 (T). After washing three times for 10 min at RT with TBST the nitrocellulose was probed o/n at 4° C with the respective primary antibody listed in Table 3. All primary antibodies were diluted in 5% (w/v) BSA in TBST. On the next day membranes were washed three times with TBST at RT and antigen-antibody complexes were detected with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Millipore) diluted in 5% (w/v) skimmed milk powder. After 1h incubation at RT membranes were washed three times with TBST and once with TBS followed by chemiluminescence detection with SuperSignal West Pico chemiluminescent substrate (Pierce) or SuperSignal West Dura extended duration substrate (Pierce) and exposure to BioMax MS film (Kodak).

Table 3 List of protein-specific and motif-specific antibodies used for immunodetection

Antibody	Antigen	Source	Working dilution	Reference
α-MPK3	AtMPK3	Dr. Klessig	1:2000	(Menke et al., 2004)
α-MPK6	AtMPK6	Dr. Klessig	1:2000	(Menke et al., 2004)
α-MPK4	AtMPK4	Dr. Klessig	1:2000	(Menke et al., 2004)
α-MKK1	AtMKK1	Dr. Nanmori	1:5000	(Matsuoka et al., 2002)
α-pTEpY	pTEpY	Millipore	1:1000	(Payne et al., 1991)

In-Gel Kinase Assay

In-gel kinase assays were performed as described by (Zhang and Klessig, 1997). Homogenized frozen leaf tissue was extracted in buffer containing 100mM Hepes pH 7.5, 5mM EDTA, 5mM glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 10mM dithiothreitol (DTT), 10mM sodium vanadate (Na_3VO_4), 10mM so-

dium fluoride (NaF), 50mM β-glycerophosphate, 1mM PMSF, 5 μg/mL antipain, 5μg/mL aprotinin, 5μg/mL leupeptin, 10% (v/v) glycerol, 7.5% (w/v) polyvinylpolypyrrolidone (PVPP). Extracts were centrifuged twice at 14,000g for 30 min at 4°C and 10μg total protein was mixed with 2X sample buffer and electrophoresed on discontinues 12.5% SDS-polyacrylamide gels embedded with 0.25mg/mL of myelin basic protein (MBP) (Sigma) in the separating gel as a substrate for the kinase. After electrophoresis, SDS was removed by washing the gel with washing buffer (25mM Tris-HCl pH 7.5, 0.5mM DTT, 0.1mM Na_3VO_4, 5mM NaF, 0.5mg/mL BSA, 0.1% (v/v) Triton X-100) three times, each for 30 min at RT. The kinases were allowed to renature in 25mM Tris-HCl, pH 7.5, 1mM DTT, 0.1mM Na_3VO_4 and 5mM NaF at 4°C o/n with three changes of buffer. The gel was then incubated at RT in 30mL reaction buffer (25mM Tris-HCl pH 7.5, 2mM EGTA, 12mM $MgCl_2$, 1mM DTT, 0.1mM Na_3VO_4) supplemented with 200nM ATP plus 50μCi γ-^{32}P-ATP (3000Ci/mmol) for 1h. The reaction was stopped by transferring the gel into 5% (w/v) trichloroacetic acid (TCA), 1% (w/v) sodiumpyrophosphate (NaPPi). Unincorporated γ-^{32}P-ATP was removed by washing in the same solution for at least 6h with five buffer changes. The gel was dried onto Whatman 3MM paper using a gel-dryer system (BioRad) and exposed to BioMax MS film (Kodak). Prestained molecular mass (Fermentas) were used to calculate the mass of kinases.

Phosphoprotein Enrichment

Total protein from *Arabidopsis* leaf tissue was extracted according to the method described by Wang et al. (2003) with modifications. Frozen tissue was ground in liquid nitrogen and transferred to a pre-chilled 15mL-tube filled up to 5mL with frozen tissue powder. Leaf powder was washed twice with 10mL ice-cold acetone and centrifuged in a swing-out rotor at 2,500g for 5 min at 4 °C. The pellet was resuspended in 10mL ice-cold 10% (w/v) TCA in acetone and transferred to an ultrasound water bath for 10 min at 4 °C. After centrifugation the pellet was washed (by fully resuspending the pellet and centrifugation as described above) 3 times with 10mL ice-cold 10% (w/v) TCA/acetone, once with 10mL ice-cold 10% (w/v) TCA and then with 10mL ice-cold 80% (v/v) acetone twice. The pellet was dried shortly on ice before resuspension at RT in 5mL freshly prepared dense SDS buffer (100mM Tris-HCl pH 8.0, 30% (w/v) Saccharose, 2% (w/v) SDS, 5% (v/v) β-MEtOH) and adding 5mL Phenol/Tris-HCl pH 8.0 (Applichem). After vigorously shaking on a vortex, the samples were centrifuged in a swing-out rotor at 5,000g for 10 min at RT. After carefully transferring the upper phase to a clean 50mL tube the samples were vortexed and 2 aliquots of 100μL each were transferred to 1.5mL eppendorf tubes. Total protein was precipitated for 30 min at -20 °C by addition of 5 volumes of ice-cold 100mM ammonium acetate in methanol to all samples. Proteins were collected by centrifugation in a fixed angle rotor at 4 °C for 10 min at 6,000g for 50mL tubes and 14,000g for 1.5mL tubes. The pellets

were then washed twice with ice-cold 100mM ammonium acetate/methanol and two times with ice-cold 80% (v/v) acetone. After shortly drying the pellets on ice, a single 100µL derived pellet was resuspended in 100µL 8M urea for determining the total protein concentration by performing a Bradford protein assay as described above.

The phosphorylated subproteome was enriched as previously described (Wolschin et al., 2005) by metal oxide/hydroxide affinity chromatography with modifications. Total protein was resolublized o/n in incubation buffer (IP150I) (30mM 2-(N-morpholino)-ethansulfonic acid (MES)-HCl pH 6.1, 150mM L-glutamine, 150mM L-aspartine, 10mM imidazole, 0.25% (w/v) 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 8M urea) on a rotator at 10 ˚C to a final concentration of 0.5mg/mL protein. After 5 min incubation in an ultrasound water bath at RT, resolublized samples were cleared by centrifugation at 12,000g for 10 min at 10˚C. Meanwhile, aluminium hydroxide (Al(OH)$_3$) was washed twice using IP150I buffer and aliquots of washed Al(OH)$_3$ were transferred to 15mL tubes. Al(OH)$_3$ was centrifugated at 3,000g for 5 min at 10 ˚C and supernatant was carefully removed before transferring 10mL of the clarified resolublized protein to 0.5g Al(OH)$_3$. Binding of the phosphoproteins to Al(OH)$_3$ was performed by incubation on a rotator at 10 ˚C for 1 h. Al(OH)$_3$ – phosphoprotein complexes were washed 6 times by resuspending in 10mL IP150I and centrifugation at 3,000g for 5 min at 10 ˚C. Elution of phosphoproteins-enriched fraction was carried out by resuspending the pellet in 5mL elution buffer (200mM potassium pyrophosphate (KPPi) pH 9.0, 8M urea) and incubation on a rotator at RT for 30 min. After centrifugation in a swing-out rotor at 5,000g at RT the supernatant was carefully transferred to a 15mL tube before protein was precipitated on ice for 1 h by adding 0.01 volumes 7% (w/v) sodium deoxycholate, vigorously shaking and subsequent addition of 0.3 volumes 100% (w/v) TCA. Phosphoproteins were collected in a 1.5mL tube by repeated centrifugation at 14,000g for 10 min at 4 ˚C before washing the protein pellet with 24% (w/v) TCA and twice with frozen (-20˚C) 80% (v/v) acetone in 10mM Tris-HCl pH 7.5. Protein pellets were stored under 80% (v/v) acetone at -20 ˚C until further use.

Isoelectric Focusing and Gel Electrophoresis

Proteins were analysed by one- or two-dimensional PAGE as described (Rohrig et al., 2006) with minor changes. For one dimensional gel electrophoresis, the samples were dissolved in NuPAGE® sample buffer (Invitrogen), heated for 20 min at 70 °C. Proteins were separated on NuPAGE® 4–12% Bis(2-hydroxyethyl)-imino (Bis)-Tris gels with MES-SDS running buffer (Invitrogen). Two-dimensional PAGE was performed using an Ettan IPGphor II isoelectric focussing (IEF) system (GE Healthcare) for the first dimension. The proteins (5–20µg) were solubilized in 125µL rehydration solution (7M urea, 2M thiourea, 2% (w/v) CHAPS, 0.2%

(w/v) DTT, 0.5% (v/v) immobilized pH gradient (IPG) buffer pH 4–7 (Invitrogen), 0.002% (w/v) bromophenol blue) for at least 1 h at RT. For isoelectric focussing, 7 cm long IPG strips, pH 4–7 (Invitrogen) were used. The strips were rehydrated for 14 h at 20 °C in rehydration solution. IEF was conducted using the following voltage steps: 30 min at 500 V, 30 min at 1000 V and, finally, 100 min at 5000 V. For the second dimension, NuPAGE® 4–12% Bis-Tris ZOOM gels (Invitrogen) were used. IPG strips were prepared for the second dimension SDS-PAGE by equilibrating them for 15 min in the NuPAGE® sample buffer (Invitrogen) with NuPAGE® sample reducing agent followed by a 15 min incubation in NuPAGE® sample buffer containing 2% (w/v) iodoacetamide.

Staining of Gels

Phosphoproteins were stained with the Pro-Q® Diamond fluorescent gel stain according to the protocol of the manufacturer (Invitrogen). After phosphoprotein staining, the gels were incubated in PageBlue™ protein staining solution (Fermentas) according to the company's protocol to monitor whether equal amounts of protein were loaded onto gels. The gels stained with fluorescent dyes were visualized using a Typhoon 9200 scanner (GE Healthcare) at an excitation wavelength of 532 nm and a bandpass emission filter of 610 nm.

In-Gel Tryptic Protein Digest, Sequence Data and Protein Identification by Mass Spectrometry

Protein spots were picked from gels stained with Pro-Q® Diamond fluorescent dye by a *Proteineer* spII spot picker and passed to a *Proteineer* dp robot (Bruker Daltonics) for automated tryptic digestion and target preparation. Samples were spotted onto Anchorchip targets (Bruker) using a thin-layer protocol with HCCA matrix (Gobom et al., 2001). MALDI MS and MS/MS analyses were performed on an Ultraflex III system (Bruker) according to the following workflow. PMF's were collected on the thin-layer samples and post-calibrated using tryptic autodigestion masses, when present. These spectra were used for database searches using Mascot 2.2 (Matrix Science) against the NCBInr database without taxonomic restrictions and with a 20ppm mass tolerance. Non-post-calibrated spectra which failed to return identifications in this round of searching were then used to search with a 100ppm mass tolerance. For each sample spot, precursor ions of sufficient intensity and quality were flagged automatically for fragmentation according to the following strategy: where a PMF-based identification was successful, two assigned precursors were selected (if present) for validation by MS/MS. Then, up to five further unassigned precursors were flagged for fragmentation. In cases where PMF-based identification failed, five precursors were selected on the basis of signal quality. All samples on the target were re-crystallized in preparation for MS/MS collection. MS/MS spectra were also used to perform Mascot MS/MS searches against the database mentioned above. Search results were

evaluated on the basis of the following criteria: mascot scores above corresponding significance thresholds (for the NCBInr database PMF`s 79, PFF`s: 47), a low mass error, a continuous open reading frame (in the case of the cDNA database). Marginal hits were accepted if both MS and MS/MS searches returned sub-threshold scores for the same protein.

Phosphatase Treatment of Phosphoproteins

Phosphoproteins (enriched from 5mg of total proteins) were separated by NuPAGE® 4-12% Bis-Tris gels (Invitrogen) and transferred to a Immobilon-P PVDF membrane (0.45 µm, Millipore). The membrane was blocked with TBS containing 0.1% (v/v) Triton X-100 and 1% (w/v) BSA for 1 h to prevent unspecific binding. Phosphatase treatment of membrane-bound proteins was performed with 800 U Lambda protein phosphatase (New England Biolabs) per ml TBS which contained 0.1% (v/v) Triton X-100, 1% (w/v) BSA and 2 mM $MnCl_2$. The incubation was carried out on a shaker for 4 hr at 25 °C. After phosphatase treatment the membrane was washed once with Phosphate-buffered saline (PBS; 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4, 2.7mM KCl, 137mM NaCl pH 7.4) and five times with H_2O (5 min each). Then the membrane was air dried, shortly immersed in methanol, subsequently treated with 7% (v/v) acetic acid/10% (v/v) methanol for 15 min and finally washed 4 times with H_2O (5 min each). To detect phosphoproteins the membrane was immersed for 15 min in Pro-Q™ Diamond Phosphoprotein Blot Stain (a 1000-fold dilution of Pro-Q™ Diamond blot stain reagent in Pro-Q™ Diamond blot stain buffer). Excess of staining solution was washed off 4 times with 30 mL 50 mM sodium acetate, pH 4, 20% (v/v) acetonitrile. After destaining the membrane was air dried and phosphoproteins were visualized using an excitation wavelength of 532 nm and measuring emission at 580 nm. To stain total proteins the membrane was immersed for 2 min in PageBlue (Fermentas) and then destained in 30% (v/v) ethanol until spots were clearly visible.

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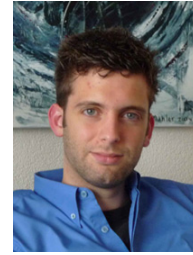
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PUBLICATIONS / FELLOWSHIPS

Research papers

1. Beckers, G.J.M. and Spoel, S.H. (2006) Fine-Tuning Plant Defense Signaling: Salicylate versus Jasmonate. ***Plant Biology,*** 8 (1): 1-10

2. Beckers, G.J.M. and Conrath, U. (2006) Microarray data analysis made easy. ***Trends in Plant Science,*** 11 (7): 322-323

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6. Beckers, G.J.M., Jaskiewicz, M., Liu, Y., Underwood, W.R., He, S.Y., Zhang, S. and Conrath, U. (2009) Mitogen-Activated Protein Kinases 3 and 6 Are Required for Full Priming of Stress Responses in *Arabidopsis thaliana*. ***Plant Cell,*** 21: 944-953

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1. Beckers, G. J. M., Gilbert, S., Schmitt, T. and Conrath, U. (2006) Priming as a mechanism of systemic acquired resistance in plants. ***Journal of Plant Diseases and Protection*** 113 (3): 137

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1. Spoel, S.H., Beckers, G.J.M., Pieterse, C.M.J. and Dong, X. (2004) NPR1 modulates salicylate- and jasmonate- dependent defense responses in plants. 15th International Conference on Arabidopsis Research, Berlin/ Germany.

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