PhD thesis
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MAP Kinase 4 substrates and plant innate immunity

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Handed in: 31/12/2015
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Preface

This thesis concludes my PhD work at the department of Biology, University of Copenhagen. My main research focus has been on substrates of the immune regulating MAP kinase 4 and their involvement in immunity. The thesis consist of:

(i) A general introduction to plant innate immunity.
(ii) A review article “MAP Kinases in Arabidopsis innate immunity” published in “Frontiers in Plant Science”. This article serves as an introduction, giving an overview on the function of MAP kinases immune signaling in Arabidopsis.
(iii) An invited review article “mRNA decay in plant immunity” under revision for publication in “Cellular and Molecular Life Science”. This article gives an overview on our current understanding of the involvement of mRNA decay in plant immunity and serves as a more comprehensive introduction to (iv).
(iv) A research article “The mRNA decay factor PAT1 functions in a pathway including MAP kinase 4 and immune receptor SUMM2” published in ”The EMBO Journal”.
(v) Two draft manuscripts regarding novel MPK4 substrates and their putative function in defense.

Aknowlegements

An era has ended and I will finally leave the PMB lab! I am sure that I will be missed and trust me; I will miss all of you. I would like to thank my main supervisor Morten Petersen and co-supervisor John Mundy for their support and guidance during my research and the opportunity to work in your lab. I will also take the opportunity to thank all the current and previous lab members for making my stay in the lab such a wonderful time. I would in particular like to thank my work wife no.1 Milena for all the interesting chitchat and for being my personal scientific oracle and my work wife no.2 Àngels (even though you left me for a real job!) for simply putting up with me and my crazy ideas. Lastly, thank you Tine for bear with me the numerous times when I’ve told you that that I’m almost on my way home and end up staying two hours extra in the lab.
Abstract

Multi-layered defense responses are activated in plants upon recognition of invading pathogens. Transmembrane receptors recognize conserved pathogen-associated molecular patterns and activate MAP kinase cascades, which regulate changes in gene expression to produce appropriate immune responses. For example, Arabidopsis MPK4 regulates the expression of a subset of defense genes via at least one WRKY transcription factor. We report here that MPK4 is found in complexes in vivo with (i) PAT1, component of the mRNA decapping machinery, (ii) AOC3, a component in the biosynthesis pathway of JA and (iii) eIF4E, a component in the translational initiation protein complex. For PAT1 and eIF4E we show that MPK4 phosphorylates specific Ser and Thr residues in vitro, and that MPK4 also phosphorylates AOC3 at an unmapped residue. Specific in vivo phosphorylation for PAT1 is shown in response to pathogen recognition, which also induce its localization to cytoplasmic processing bodies. All three proteins; PAT1, AOC3 and eIF4E also interacts with MPK4 in vivo although the functional outcome of these interactions are still elusive. The thesis comprise a general introduction to plant innate immunity followed by two review articles “MAP kinase cascades in Arabidopsis innate immunity” published in *Frontiers in Plant Science* and “mRNA decay in plant immunity” under revision for *Cellular and Molecular Life Science*. Together these sections gives a comprehensive overview of *Arabidopsis* defense signaling. The results are presents as one manuscript “The mRNA decay factor PAT1 functions in a pathway including MAP kinase 4 and immune receptor SUMM2” published in *The EMBO Journal* and two draft manuscripts summarizing our data on regarding AOC3 and eIF4E in respect to MPK4.
Sammendrag

Flere forsvarslag aktiveres i planter ved genkendelse af invaderende patogener. Transmembrane receptorer genkender konserverede patogen-associeret molekylære mønstre og aktivere MAP-kinase kaskader, som regulerer ændringer i gen-ekspression for at producere passende immunresponse. For eksempel, Arabidopsis MPK4 regulerer ekspressionen af en undergruppe af forsvarsgener via mindst en WRKY transkriptionsfaktor. Vi rapporterer her, at MPK4 findes i komplekser in vivo med (i) PAT1, en komponent i "mRNA decapping", (ii) AOC3, en komponent i biosyntesevejen af JA og (iii) eIF4E, en komponent i translationsinitierings komplekset. For PAT1 og eIF4E viser vi, at MPK4 phosphorylerer specifikke Ser og Thr aminosyrerestrre in vitro, og at MPK4 også phosphorylerer en ukendt aminosyreester i AOC3. Specifik in vivo phosphorylering af PAT1 sker som reaktion på patogen genkendelse, som derudover også inducerer dens lokaliserings til cytoplasmiske "procesing bodies". Alle tre proteiner; PAT1, AOC3 og eIF4E interagerer med MPK4 in vivo, selvom det funktionelle resultat af disse interaktioner stadig er ukendte.

Afhandlingen omfatter en generel introduktion til at planters medfødte immunforsvar efterfulgt af to review-artikler " MAP kinase cascades in Arabidopsis innate immunity " offentliggjort i Frontiers in Plant Science og "mRNA decay in plant immunity" under revision for Cellular and Molecular Life Science. Disse afsnit giver tilsammen et samlet overblik over Arabidopsis forsvar signalering. Resultaterne er præsenteret som et manuskript " The mRNA decay factor PAT1 functions in a pathway including MAP kinase 4 and immune receptor SUMM2" offentliggjort i The EMBO Journal og to udkast til manuskripter som opsummerer vores viden vedrørende AOC3 og eIF4E i forhold til MPK4.
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<tr>
<td>4E-BP</td>
<td>eIF4E BINDING PROTEIN</td>
</tr>
<tr>
<td>ACD</td>
<td>ACCELERATED CELL DEATH</td>
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<td>AGO</td>
<td>ARGONAUTE</td>
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<tr>
<td>AOC</td>
<td>ALLENE OXIDE CYCLASE</td>
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<td>AOS</td>
<td>ALLENE OXIDE SYNTHASE</td>
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<tr>
<td>ARF</td>
<td>AUXIN RESPONSE FACTOR</td>
</tr>
<tr>
<td>At</td>
<td><em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>BAK1</td>
<td>BRI1-ASSOCIATED RECEPTOR KINASE1</td>
</tr>
<tr>
<td>BIK1</td>
<td>BOTRYTIS-INDUCED KINASE1</td>
</tr>
<tr>
<td>CC</td>
<td>Coiled-coiled</td>
</tr>
<tr>
<td>CDPK</td>
<td>Ca2+ dependent kinase</td>
</tr>
<tr>
<td>ClYVV</td>
<td><em>Clover Yellow Vein Virus</em></td>
</tr>
<tr>
<td>Col</td>
<td><em>Columbia</em></td>
</tr>
<tr>
<td>CSD</td>
<td>COPPER/ZINC SUPEROXIDE DISMUTASE</td>
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<tr>
<td>CTR</td>
<td>Constitutively Triple Response</td>
</tr>
<tr>
<td>DCL1</td>
<td>DICER-LIKE 1</td>
</tr>
<tr>
<td>DCP1</td>
<td>DECAPPING 1</td>
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<tr>
<td>EBF</td>
<td>EIN3 binding F-box protein</td>
</tr>
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<td>EDS1</td>
<td>ENHANCED DISEASE SUSCEPTIBILITY1</td>
</tr>
<tr>
<td>EFR</td>
<td>EF-TU RECEPTOR</td>
</tr>
<tr>
<td>eIF</td>
<td>eukaryotic INITIATION FACTOR</td>
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<tr>
<td>EIN</td>
<td>Ethylene Insensitive</td>
</tr>
<tr>
<td>ERF</td>
<td>Ethylene Response Factor</td>
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<tr>
<td>ET</td>
<td>Ethylene</td>
</tr>
<tr>
<td>ETI</td>
<td>Effector Triggered Immunity</td>
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<tr>
<td>FLS2</td>
<td>FLAGELLIN-SENSITIVE2</td>
</tr>
<tr>
<td>HR</td>
<td>Hypersensitivity response</td>
</tr>
<tr>
<td>JA</td>
<td>Jasmonic acid</td>
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<td>LAZ</td>
<td>LAZARUS</td>
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<tr>
<td>Ler</td>
<td><em>Landsberg</em></td>
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<tr>
<td>LMM</td>
<td>Lesion Mimic Mutant</td>
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<tr>
<td>LOX</td>
<td>LIPOXYGENASE</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MBP</td>
<td>MYELIN BASIC PROTEIN</td>
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<tr>
<td>MKS1</td>
<td>MAP KINASE 4 SUBSTRATE 1</td>
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<tr>
<td>MNK</td>
<td>MAPK-INTERACTING KINASE</td>
</tr>
<tr>
<td>MAPK</td>
<td>MAP Kinase</td>
</tr>
<tr>
<td>MAP2K</td>
<td>MAP Kinase Kinase</td>
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<tr>
<td>MAP3K</td>
<td>MAP Kinase Kinase Kinase</td>
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<tr>
<td>MPK4</td>
<td>MAP KINASE 4</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NDR1</td>
<td>NONRACE-SPECIFIC DISEASE RESISTANCE1</td>
</tr>
<tr>
<td>NMD</td>
<td>Non-sense Meadiated Decay</td>
</tr>
<tr>
<td>NPR</td>
<td>NON-EXPRESSOR OF PR-GENES</td>
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<tr>
<td>OPDA</td>
<td>Oxo-phytodienoic acid</td>
</tr>
<tr>
<td>OPR</td>
<td>OPDA REDUCTASE</td>
</tr>
<tr>
<td>PAD</td>
<td>PHYTOALEXIN DEFICIENT</td>
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<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
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<tr>
<td>PABP</td>
<td>poly(A) binding protein</td>
</tr>
<tr>
<td>PAT</td>
<td>PROTEIN ASOiated WITH TOPOISOMERASE-II</td>
</tr>
<tr>
<td>PBs</td>
<td>Processing Bodies</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PR</td>
<td>Pathogenesis-Related</td>
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<tr>
<td>PRR</td>
<td>Pattern Recognizing Receptor</td>
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<tr>
<td>pst</td>
<td>Pseudomonas syringae pv. tomato</td>
</tr>
<tr>
<td>PTI</td>
<td>PAMP Triggered Immunity</td>
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<tr>
<td>PTC</td>
<td>Premature termination codon</td>
</tr>
<tr>
<td>PTGS</td>
<td>Post Transcriptionally Gene Silencing</td>
</tr>
<tr>
<td>RBOHD</td>
<td>RESPIRATORY BURST OXIDASE HOMOLOG D</td>
</tr>
<tr>
<td>R-gen</td>
<td>Resistance gene</td>
</tr>
<tr>
<td>R-protein</td>
<td>Resistance Protein</td>
</tr>
<tr>
<td>RIN4</td>
<td>RPM1-INTERACTING PROTEIN4</td>
</tr>
<tr>
<td>RIPK</td>
<td>RPM1-INDUCED PROTEIN KINASE</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPM1</td>
<td>RESISTANCE TO PSEUDOMONAS SYRINGAE</td>
</tr>
<tr>
<td>RPS2</td>
<td>RESISTANCE TO PSEUDOMONAS SYRINGAE2</td>
</tr>
</tbody>
</table>
SA       Salicylic acid
SAR      Systemic Acquired Resistance
SID2     ISOCHORISMATE SYNTHASE1
siRNA    Small interfering RNA
SP       Serine-Proline
SUMM2    SUPPRESSOR of MKK1 MKK2 2
TAL      Transcription Activator-Like
TF       Transcription factor
TIR      Toll and Interleukin-1 Receptor homology
TP       Threonine-Proline
VCS      VARICOSE
WT       Wild type
Xoo      Xanthomonas oryzae pathovar oryzae
XRN      Exoribonuclease
Plant innate immunity

Plants convert sunlight and atmospheric carbon dioxide into chemical energy in the form of carbohydrates (Lodish et al., 2000). Thus, plants are a source of nutrient for numerous organisms. It is logical to assume that plants defend themselves against pathogens or herbivores to avoid depletion of their nutrients. Plants have therefore developed various defense mechanisms to avoid hosting malevolent organisms (Katagiri and Tsuda, 2010). All biological processes, including defense responses, are energy consuming, thus a tight regulation of defense can avoid superfluous use of scarce resources. Defense responses are spatial and temporal regulated, in most cases by direct or indirect recognition of pathogens. For example, plants can instigate defense by direct recognition of bacterial flagellin or indirectly by recognizing host tissue damaged by pathogens or herbivores (Gómez-Gómez and Boller, 2000; Krol et al., 2010). Plants have adapted both a mechanical defense system in the form of structural fortification of the cell wall and active defense system in form of both transmembrane and cytoplasmic immune receptors that upon activation boosts defense responses (Boller and He, 2009; Malinovsky et al., 2014). The role of the rigid cell wall and regulation of its components in response to pathogens are reviewed by Malinovsky et al (2014) and will not be covered here whereas a general introduction to immune receptors and their activation are described below.

Pathogen associated molecular patterns (PAMPs)

PAMP is a collective term used to describe conserved molecular patterns found in microorganisms that on their own is enough to induce defense responses. PAMPs are recognized by transmembrane immune receptors located in the plasma membrane. The recognition of bacterial flagellin by the pattern recognition receptor (PRR) FLAGELLIN-SENSING 2 (FLS2) in Arabidopsis is one of the best characterized PAMP recognition systems in plants (Boller and Felix, 2009; Gómez-Gómez and Boller, 2000). Another example is the perception of bacterial elongation factor-Tu by the EF-Tu (EFR1) receptor (Kunze et al., 2004; Zipfel et al., 2006). Whereas FLS2 is widely conserved across plant species, EFR belongs only to the Brassicaceae family (Zipfel et al., 2006). Transgenic expression of Arabidopsis EFR in species outside the Brassicaceae family make plants sensitive to EF-Tu induced immunity (Lacombe et al., 2010). Thus, signaling downstream of PRRs is likely well conserved across species.
Both EFR and FLS2 hetero dimerizes with the co-receptor BRII-ASSOCIATED KINASE 1 (BAK1) in a ligand induced interaction (Chinchilla et al., 2007; Roux et al., 2011; Schulze et al., 2010). Phosphorylation of BOTRYTIS-INDUCED KINASE 1 (BIK1) is induced upon activation of both FLS2 and EFR (Lu et al., 2010; Zhang et al., 2010). In Arabidopsis BIK1 resides in a complex with both BAK1 and FLS2 or EFR and upon PAMP perception BIK1 is rapidly phosphorylated which trigger BIK1 to activate downstream immune responses (Lu et al., 2010; Zhang et al., 2010).

One of the early effects of PAMP triggered immunity (PTI) is the generation of reactive oxygen species (ROS) in an oxidative burst producing apoplastic superoxide ($O_2^-$), which can be converted into hydrogen peroxide ($H_2O_2$) (Torres et al., 2006). The transmembrane RESPIRATORY BURST OXIDASE HOMOLOG D (ROBHD) is a key enzyme in producing superoxide, it oxidize intracellular NADPH and transports the electron to the apoplastic space where it reacts with molecular oxygen producing superoxide (Torres et al., 2005, 2006). Production of ROS, like hydrogen peroxide is directly toxic to pathogens and it induce cell wall strengthening while also functioning as a systemic signaling molecules (Kadota et al., 2015; Miller et al., 2009). Recent studies have shown that ROBHD sits in a complex with FLS2/EFR, BAK1 and BIK1, when PTI is induced, BIK1 directly phosphorylates resides in RBOHD. When hindering this phosphorylation RBOHD is not activated properly resulting in increased susceptibility against non-adapted pathogens (Kadota et al., 2014; Li et al., 2014).

In general, PTI is relatively mild and mostly effective against non-adapted pathogens that do not employ effector molecules evolved to circumvent PTI signaling. In addition to ROS signaling other canonical signaling pathways include mitogen activated protein kinases (MAPKs) that for example can adjust transcript accumulation of defense related genes. The involvement of MAPKs in PTI is reviewed in more details in “MAP Kinases in Arabidopsis innate immunity”.

**Pathogenic effector molecules and host resistance (R) proteins**

Successful pathogens have evolved effector proteins or molecules that can modify the host and support growth of the pathogen. Bacterial pathogens deliver these effectors by types of secretion systems that can effectively inject effector proteins directly into the host cell. Mutations that disrupt the type-three secretion system in gram-negative bacterial pathogens are often accompanied by weakened virulence and are inadequate of overcoming PTI (Cunnac et al., 2009).
Effector proteins function in numerous ways. One example is Transcription Activator-Like (TAL) effectors functioning as transcription factors, which bind the promoter of specific host genes and induce their transcription. For effective infection in rice the pathogen *Xanthomonas oryzae pathovar oryzae* (*Xoo*) deploy the TAL effector *pthXo1* (Yang et al., 2006). *PthXo1* binds the promoter and induce the induction of *OsSWEET11*, which is important for *Xoo* infection (Chen et al., 2010; Yang et al., 2006; Yuan et al., 2010). *OsSWEET11* is presumably a sugar transporter that induce sugar efflux in favor of the pathogen (Chen et al., 2010), however it has also been suggested that *OsSWEET11* functions as a cupper transporter that reduce the cupper concentration in the xylem sap to facilitate infection of the cupper sensitive *Xoo* (Yuan et al., 2010). In *Arabidopsis* infection with *Pseudomonas* (*Pst*) DC3000 induce accumulation of transcripts from 7 *SWEET* genes, whereas infections with the *Pst* DC3000 *HrcU* mutant which cannot inject type three-effector proteins into the host did not induce accumulation of three of these transcript (Chen et al., 2010). Plants resistant to pathogens deploying specific TAL effectors have evolved defense genes that are under control of promoters mimicking that of the TAL effector targets, which thereby are transcribed in presence of the TAL effectors inducing a defense response (Hutin et al., 2015).

Effector proteins can also modify host proteins to quench host defense responses. RPM1 INTERACTING PROTEIN 4 (RIN4) is one of the best characterized targets of bacterial effectors. RIN4 is anchored to the plasma membrane and resides in complex with or are in close proximity to FLS2 and activation of FLS2 induces phosphorylation of RIN4 important for full activation of PTI (Chung et al., 2014; Qi et al., 2011). Although the molecular function of RIN4 is not fully understood, its role in immunity is emphasized by being a target of no less than four bacterial effectors comprising AvrRpm1, AvrB, AvrRpt2 and HopF2 while being a guardee of the two R proteins RPM1 and RPS2 in *Arabidopsis* (Axtell and Staskawicz, 2003, 2; Mackey et al., 2002, 2003; Selote and Kachroo, 2010; Wang et al., 2010; Wilton et al., 2010). These effector proteins and conjugant R proteins function by different mechanisms. AvrRpt2 functions as a protease and when introduced into the host it degrades RIN4, the lack of RIN4 in turns activate RPS2 causing effector triggered immunity (ETI) (Axtell and Staskawicz, 2003; Mackey et al., 2003, 4). PTI and ETI signaling is somewhat similar although ETI responses are more prolonged and pronounced and can include localized cell death (Cui et al., 2014). *Arabidopsis rps2* loss-of-function mutants are more susceptible to AvrRpt2 expressing pathogens, thus it is likely that resistant plants have evolved RPS2 in response AvrRpt2 mediated bacterial virulence to restore resistance (Afzal et al., 2011).
AvrB and AvrRpm1 on the other hand mediate RIN4 phosphorylation through RPM1-INDUCED PROTEIN KINASE (RIPK). FLS2 and RIPK mediated RIN4 phosphorylation occurs on different residues and whereas FLS2 facilitates PTI through RIN4, RIPK mediated phosphorylation dampens PTI. In Arabidopsis the R protein RPM1 sense RIPK mediated phosphorylation of RIN4 and instigate ETI (Chung et al., 2011, 2014; Liu et al., 2011).

The fourth effector protein HopF2 inhibits accumulation of PAMP induced phosphorylated RIN4, dampening PTI (Chung et al., 2014). Besides its interaction with RIN4, HopF2 also targets the FLS2 co-receptor BAK1 and BIK1, thus HopF2 reduced accumulation of PAMP induced phosphorylation of RIN4 is possible to occur at multiple levels (Chung et al., 2014; Wang et al., 2010; Zhou et al., 2014). Expression of HopF2 in Arabidopsis dampens PTI and is not associated with activation of ETI, therefore HopF2 represents a functional effector protein apparently not detected by Arabidopsis R proteins.

Detection of AvrB, AvrRpm1 and AvrRpt2 modified RIN4 by RPM1 and RPS2 are examples of indirect recognition of effector proteins. In this model host proteins are guarded by R proteins that detect modification of their guardees (Dangl and Jones, 2001; Van Der Biezen and Jones, 1998).

Some R proteins directly recognize effector proteins and trigger ETI, this is true for Arabidopsis R protein RRS1 which directly recognize the Ralstonia solanacearum effector protein AvrPopP2 (Deslandes et al., 2003).

A third class of R proteins has evolved as decoys, mimicking potential host effector targets. This hypothesis was first suggested by Hoorn and Kamoun (2008). A direct example of this is the Arabidopsis R protein RRS1 that contain a WRKY domain normally associated with transcription factors. The R. solanacearum effector protein PopP2 directly acetylates a group of defense related WRKY transcription factors inhibiting their DNA binding capabilities. PopP2 also bind the decoy RRS1, which is paired with another R protein, RPS4 and when PopP2 bind the RRS1 decoy the RRS1/RPS4 complex activate ETI (Le Roux et al., 2015; Sarris et al., 2015).

**Autoimmunity**

HR is often associated with ETI and is typically characterized by rapid, localized programmed cell death at the site of pathogen invasion (Mur et al., 2008). Evidence linking ETI to HR include; (i) presence of paired effectors and R proteins (ii) over-expression of R proteins and (iii) expression of auto-active R proteins are all sufficient to induce HR (Gao et al., 2011; Grant et al., 2000; Peart et al., 2005; Stokes et al., 2002). Programmed cell death during HR seems to rely on light dependent
ROS production in the chloroplasts as inhibition or induction of chloroplast ROS production respectively delay or increase cell death when applied with virulent pathogens (Zurbriggen et al., 2010). Pathogen induced HR also appear to depend on autophagy as autophagy deficient mutants do not display full HR (Hofius et al., 2009; Munch et al., 2015). It is however still unclear whether ETI triggered HR is cause or a consequence of disease resistance. Some evidence support HR as a cause of ETI against strictly biotrophic pathogens (Wang et al., 2011). Whereas other findings support that HR can be uncoupled from ETI (Bendahmane et al., 1999; Bulgarelli et al., 2010; Coll et al., 2010; Heidrich et al., 2011; Munch et al., 2015).

Autoimmune mutants display constitutive ETI phenotypes. We have previously characterized the autoimmune mutants accelerated cell death 11 (acd11) and map kinase 4 (mpk4) (Brodersen et al., 2002; Petersen et al., 2000). ACD11 is a putative sphingolipid transfer protein, but its precise role during these processes is still unknown. acd11 is a so called lesion mimic mutants as it exhibits constitutive defense responses and cell death without pathogen perception (Brodersen et al., 2002; Palma et al., 2010). Activation of the R protein LAZARUS 5 (LAZ5) is responsible for the acd11 phenotype. LAZ5 is activated in absence ACD11, although the mechanism of this detection remains unknown, but laz5 loss-of-function mutants completely rescue the lesion mimic phenotype (Palma et al., 2010). The mpk4 mutant similarly exhibits autoimmunity caused at least by activation of the R protein SUPPRESSOR OF MKK1 MKK2 2 (SUMM2) (Petersen et al., 2000; Zhang et al., 2012). The function of MPK4 is discussed in detail in “MAP Kinases in Arabidopsis innate immunity”. It is clear that Arabidopsis loss-of-function mutants shadowed by autoimmune phenotypes are often the result of inappropriate activation or R-proteins. However, since these mutants constitutively exhibits immune responses they are often categorized as negative regulators of defense due to pleiotropic effects of ETI (Rodriguez et al., 2015). It is therefore important to elucidate this issue when investigating the true in vivo function of these proteins.

References


MAP kinase cascades in Arabidopsis innate immunity
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INTRODUCTION

Plants have evolved an effective basal defense system to detect and limit the growth of pathogens. Pathogens may be recognized by the host via the perception of conserved microbial structures termed pathogen-associated molecular patterns (PAMPs). PAMPs are recognized via transmembrane pattern recognition receptors (PRRs) that bind specific PAMPs and initiate intracellular immune responses (Zipfel et al., 2008). These PAMP-triggered immunity (PTI) responses include the generation of reactive oxygen species (ROS), extracellular alkalization, and protein phosphorylation with associated gene regulation that ultimately restricts the growth of the microbial intruder (Cuerva-Ibanez and Ratcliffe, 2010).

Mitogen-activated protein kinase (MAPK) signaling plays central roles in such intracellular immunity pathways. In general, MAP kinase signaling is initiated by the stimulus-triggered activation of a MAP kinase kinase kinase (MAP3K), followed by MAP kinase kinase (MAP2K) activation, which may be directly or indirectly effected by a PRR, in turn leads to the phosphorylation and activation of downstream MAP kinase kinases (MAP3K; also called MEKK). Subsequently, the MAP2K phosphorylates the downstream MAPK sequentially leading to changes in its subcellular localization and/or phosphorylation of downstream substrates including transcription factors which alter patterns of gene expression (see Figure 1). General functions of MAPK cascades in plant biology have recently been reviewed elsewhere (Füll et al., 2009; Rodriguez et al., 2010; Komis et al., 2011).

MAPK CASCADES IN PTI

A few PRRs have been documented to stimulate MAPK signaling upon perception of PAMPs. These include the flagellin receptor FLS2 (Felix et al., 1999; Gomez-Grines and Boller, 2000), the bacterial elongation factor EF-Tu receptor EFR (Zipfel et al., 2006), and the chitin receptor CERK1 (Miya et al., 2007). The Arabidopsis genome encodes 60 MAP3Ks, 10 MAP2Ks, and 20 MAPKs (Ichimura et al., 2002). This indicates that MAPK cascades may not simply consist of single MAP3Ks, MAP2Ks, and MAPKs connected together. Instead, it suggests that there is some level of redundancy, and that the spatial and temporal activities of different components may be strictly regulated to minimize unwanted cross-talk. The three MAPKs MPK3, MPK4, and MPK6 are the most intensively studied plant MAPKs, and all three were implicated in defense signaling a decade ago (Petersen et al., 2000; Asai et al., 2002). MPK11, a close homolog to MPK4, has also recently been shown to be activated by PAMP treatment (Betheke et al., 2012).

MPK3, MPK4, and MPK6 are all activated by PAMPs such as flg22 (a conserved 22 amino acid flagellin peptide) and elf18 (elongation factor-Tu peptide; Felix et al., 1999; Zipfel et al., 2006). However, these three MAPK cascades are differently regulated already at the PRR level. For example, the two receptor kinases BAK1 and BKK1 genetically regulate PAMP signaling through their interactions with cognate PRRs (Roux et al., 2011; Schwessinger et al., 2011). The BAK1 mutant allele bak1-5 carries a Cys408Tyr substitution adjacent to its kinase catalytic loop. This impairs its flg22-regulated kinase activity and inhibits phosphorylation of MPK4. However, the catalytic complex formed between mutant BAK1 in bak1-5 and FLS2 is still able to induce phosphorylation of MPK3/MPK6 (Roux et al., 2011; Schwessinger et al., 2011). Interestingly, MPK3/MPK6 phosphorylation was impaired in only the double bak1-5 bkk1 background and not in the individual bak1-5 and bkk1 lines (Roux et al., 2011).

Asai et al. (2002) developed an elegant protoplast expression system in an attempt to identify signaling components downstream of FLS2. With this system they were able to show a complete MAPK cascade downstream of FLS2 consisting of the MAP3K MEKK1, two MAP2Ks (MKK4 and MKK5), and the MAPKs MPK3/MPK6. However, genetic evidence later showed...
MAP kinase cascades in immunity

**FIGURE 1** | (A) MAPK signaling cascades are attractive targets for bacterial effectors. The *P. syringae* HopAI1 effector irreversibly inactivates MPK4 to prevent immune responses. The R protein SUMM2 may guard processes downstream of MPK4 independent from MKS1, and triggers a hypersensitive response in the event of loss or inactivation of MPK4. (B) PAMP perception by PRRs instigates a signaling cascade, often via co-receptors, which causes activation of MAP3K MEKK1 and two MAP2Ks MKK1 and MKK2. These phosphorylate and activate MPK4 which then phosphorylates its substrate MKS1, releasing MKS1 in complex with WRKY33. MKP2/MKP6 sequentially phosphorylate WRKY33 allowing it to promote PAD3 transcription, thus activating plant defense.

that MEKK1 kinase activity was dispensable for MPK3/MPK6 activation, although mkk1 plants were impaired in MPK4 activation (Rodriguez et al., 2007). Interestingly, expressing a kinase dead version of MEKK1 in mok1 plants completely restored the activation of MPK4 upon treatment with flg22, suggesting that MEKK1 may “simply” act as a scaffold protein (Rodriguez et al., 2007). Biochemical and genetic studies further revealed that the two MAP2Ks MKK1 and MKK2 interact with both MEKK1 and with MPK4, and that flg22-induced MPK4 activation is impaired in the double mkk1 mkk2 mutant. This indicates that MKK1 and MKK2 are partially redundant in MPK4 mediated downstream signaling (Gao et al., 2008; Qiu et al., 2008b).

**MAP CASCADES IN EFFECTOR-TRIGGERED IMMUNITY**

In addition to PTI plants also employ resistance (R) proteins as cytoplasmic receptors to directly or indirectly recognize specific pathogenic effector proteins injected into host cells as virulence factors. Effector-triggered immunity (ETI) and PTI share a number of responses, although ETI also includes varying levels of rapid, localized cell death in what is called the hypersensitive response. R protein-dependent recognition initiates immune responses in ETI. R proteins may recognize effector proteins either directly or indirectly by monitoring changes in the effector’s host target(s). This latter case gave rise to the guard hypothesis in which R proteins guard host guardees that are manipulated by pathogen effectors (Van Der Biezen and Jones, 1998). The genetic characterization of the MEKK1/MKK1–MKK2/MPK4 cascade as a negative regulatory pathway of defense responses was at odds with the activation of the pathway by PAMPs. Instead, it was possible that the severe phenotypes of the kinase knockout mutants were caused by activation of one or more R protein(s) guarding this kinase pathway. Indeed, in an elegant screen for suppressors of the mkk1 mkk2 double mutant, Zhang et al. (2012) identified the R protein SUMM2 (suppressor of mkk1 mkk2). The T-DNA insertion line summ2-8 completely suppressed the severe mkk1 mkk2 phenotype in respect to morphology, cell death, ROS levels and PR gene expression (Zhang et al., 2012). The analogous knockout phenotype of the upstream MAP3K MEKK1 is also completely suppressed in the summ2-8 background. Interestingly, although the mpk4 mutant shares a similar phenotype with the knockouts of its upstream kinase partners, the mpk4 phenotype is not fully suppressed by the summ2-8
mutation, as double mpk4 sum2-8 mutants still retain residual cell death and low levels of ROS. This suggests that MPK4 is involved in other pathways independent of SUMM2, and that MPK4 may be guarded by additional R proteins (Zhang et al., 2012; Figure 1A).

The importance of MAPK signaling in immunity is emphasized by studies reporting bacterial effector proteins targeting MAPK cascades for downregulation (Zhang et al., 2007a,b, 2012; Cui et al., 2010). For example, the Pseudomonas syringae effector protein HopA1 targets and irreversibly inactivates MPK3, MPK4, and MPK6, thereby suppressing immune responses which would otherwise inhibit bacterial growth (Zhang et al., 2007a, 2012). In addition, the P. syringae effector protein AvrB has been shown to interact with and induce the phosphorylation of MPK4, although it has not been shown if this phosphorylation occurs as a direct effect of AvrB action or via recognition of AvrB by the plant immune system (Cui et al., 2010).

In plants carrying functional SUMM2 alleles, immune responses are activated by bacterial effector proteins targeting the MPK4 pathway (Figure 1A). For example, inducible expression of the bacterial HopA1 effector in wild-type plants gives rise to a defense phenotype similar to that seen in mkk1, mkk3 mkk2, and mpk4 mutants including elevated levels of ROS, PR gene expression, and cell death (Zhang et al., 2012). SUMM2 apparently does not interact directly with the kinase components of the MEKK1/MEKK1-MKK2/MPK4 signaling cascade, suggesting that SUMM2 most likely guards a downstream target of MPK4 activity (Zhang et al., 2012). At present, the best studied in vivo substrate of MPK4 activity is MPK4 substrate 1 (MKS1) which forms a nuclear complex with MPK4 and the WRKY33 transcription factor (Andreason et al., 2005; Qiu et al., 2008a). Phosphorylation of MKS1 follows MPK4 activation by flg22 perception and, once phosphorylated, MKS1 is released from complexes with MPK4, thereby releasing the WRKY33 transcription factor to bind to its cognate target genes (Qiu et al., 2008a). It has therefore been proposed that MPK4 and MKS1 sequester WRKY33 in the absence of pathogens, and free WRKY33 to induce resistance upon pathogen perception (Figure 1B, left).

As MKS1 is the only known direct target of MPK4, Zhang et al. (2012) tested whether MKS1 interacted with the R protein SUMM2 which seemingly guards MPK4 activity. However, no interaction between SUMM2 and MKS1 was detected. Since mkk1 mutants have a wild-type growth phenotype, and the mpk4 phenotype is strongly suppressed in the mkk1 background, SUMM2 may guard a process downstream of MPK4 that is independent of MKS1 (Petersen et al., 2010).

WRKY TRANSCRIPTION FACTORS

The plant-specific WRKY family is a large group of transcription factors which bind a conserved W-box sequence in the promoters of numerous genes including those encoding PR proteins. WRKY33 was found to induce the transcription of PHYTOALEXIN DEFICIENT 3 (PAD3), which encodes the cytochrome P450 monooxygenase 7B15 required for synthesis of the antimicrobial compound camalexin (Zhao et al., 1999; Qiu et al., 2008a; Figure 1B). The wrky33 mutant exhibits enhanced susceptibility toward necrotrophic pathogens such as Botrytis cinerea, while WRKY33 overexpression results in increased resistance due to enhanced PAD3 expression (Zhang et al., 2006).

MPK3 and MPK6 activities also induce the production of camalexin. Transient overexpression of the constitutively active, phospho-mimic mutant forms of MKK4/MKK5 (MKK4DD and MKK5DD), which are the upstream MAP2Ks of MPK3/MPK6, has been reported to induce transcription of both PAD2, which encodes γ-glutamylcysteine synthetase functioning in glutathione biosynthesis, and PAD3. Both PAD2 and PAD3 are necessary for camalexin production (Parry et al., 2007; Ren et al., 2008). Pathogen-induced camalexin accumulation is partially comprised in mpk3 but not notably in mpk6 mutants, yet camalexin accumulation in mpk3 mpk6 double mutants is almost completely abolished (Ren et al., 2008). While this implicates MPK3/MPK6 in camalexin synthesis, caution should be applied in evaluating results obtained from the mpk3 mpk6 double mutant as it is arrested at the cotyledon stage and is unable to initiate true leaves (Wang et al., 2007). Upstream of MPK3/MPK6 in camalexin induction, MKK4 and MKK5 are activated by the MAP3Ks MEKK1 and MAPKKKs in response to fungal pathogens (Ren et al., 2008). Yet another MAP2K, MKK9, whose upstream MAP3K(s) remains unidentified, is also involved in MPK3/MPK6 activation, as plants expressing phospho-mimic MKK9DD produce even more camalexin than plants expressing MKK4DD or MKK5DD (Xu et al., 2008).

To delineate the link between MPK3/MPK6 activation and camalexin accumulation, Mao et al. (2011) elegantly introduced the phospho-mimic mutant NMEKK2DD, an MKK4 and/or MKK5 ortholog from Nicotiana tabacum, into an array of different wrky mutants in a search for essential transcription factors involved in MPK3/MPK6 mediated camalexin induction. Interestingly, NMEKK2DD was able to induce camalexin accumulation in all tested mutant lines except wrky33. In addition, WRKY33 proved to be a substrate of MK3/MPK6 activity, and overexpression of non-phosphorylatable forms of WRKY33 could not fully complement the inability of wrky33 mutants to express PAD3 and accumulate camalexin (Mao et al., 2011; Figure 1B, right).

WRKY33-induced PAD3 expression therefore appears to involve both MPK4- and MPK3/MPK6-mediated signaling (Andreason et al., 2005; Qiu et al., 2008a; Mao et al., 2011). Mao et al. (2011) proposed a model in which PAD3-mediated camalexin induction occurs differentially depending on the type of pathogen causing the immune response. In this model, bacterial pathogens induce an MPK3 mediated response while fungal pathogens initiate an MPK3/MPK6 mediated response. This hypothesis is based on overexpression of the constitutively active MKK4/MKK5 ortholog NMEKK2DD, rendering MPK3/MPK6 hyperactive and able to induce PAD3 expression (Mao et al., 2011). In support of this hypothesis, the mpk3 mpk6 double mutant is comprised in N. cinnerea-induced PAD3 induction (Ren et al., 2008). Nonetheless, and as noted above, some care should be taken with experiments based on mpk3 mpk6 double mutants given their developmental lethality (Wang et al., 2007).

An alternative model may therefore be proposed which combines the MPK4 and MPK3/MPK6 pathways into a dual control of PAD3 regulation in response to pathogen perception (Figure 1B). In such a model, WRKY33 is sequestered in a nuclear complex...
comprising at least MPK4 and MKS1 in unchallenged plants, and is released following PAMP perception (Qi et al., 2008a). Phosphorylation is dispensable for WRKY33 to bind its cognate W-box cis-elements, although it does promote transcriptional activation (Mao et al., 2011). This is illustrated by the fact that PAE3 expression is induced in mpk4 plants (Qi et al., 2008a), perhaps due to the basal activity of free non-phosphorylated WRKY33 or by free WRKY33 activated by basal MPK3 and/or MPK6 activity. In this scenario, once WRKY33 is released from its nuclear complex with MPK4 and MKS1, it is phosphorylated and hence activated by MPK3/MPK6, thereby inducing camalexin levels through PAE3 expression. The elevated PAE3 expression induced from NMeK2250 hyper-activated MPK3/MPK6 (Mao et al., 2011) is not in conflict with this model, as it is likely that hyperactive MPK3/MPK6 are able to phosphorylate residual free WRKY33, thus bypassing other possible feedback mechanisms in PAE3 expression.

In this model, MPK4 and MPK3/MPK6 function together as a binary switch conferring dual regulation. Clarification of the mode of action in which MPK4 and MPK3/MPK6 function clearly needs further elucidation and should include experiments using catalytically inactive and/or inactivatable MPK4 (Petersen et al., 2008; Brodersen et al., 2006). Application of fungal PAMPs to plants expressing catalytically inactive MPK4 might indicate whether phosphorylation of free WRKY33 by endogenous MPK3/MPK6 is enough to induce expression of PAE3.

MAPK IN GENERAL STRESS SIGNALING
The refined work of Popescu et al. (2009) identified a MAP2K–MAPK phosphorylation network covering 570 MAPK substrates by combinatorially pairing active MAP2Ks with MAPKs, and then subjecting them to a protein microarray phosphorylation assay. Interestingly, the substrates identified were enriched for transcription factors involved in stress responses. Notably, MPK6 phosphorylated 32% of the identified targets, of which 40% overlapped with MPK3 targets (Popescu et al., 2009). This is in agreement with earlier data, similarly obtained from a protein microarray study (Feilner et al., 2008). Equally noteworthy is the finding that MPK3 also shared 30% of its targets with MPK4, revealing intensive synergy in MAPK signaling (Popescu et al., 2009).

In addition to MAPK cascade, ROS also play a pivotal role in stress signaling (Rodriguez et al., 2008). Ox1 is a serine/threonine kinase induced by general ROS-generating stimuli, is required for full activation of MPK3/MPK6 after treatment with H2O2 (Rentel et al., 2004). Although Ox1 is characterized as an upstream regulator of MPK3 and MPK6 activation, MPK3/MPK6 have been shown to phosphorylate Ox1 in vitro. This suggests that there is a feedback loop, but in vivo data supporting such a loop has not been shown (Forzani et al., 2011).

In addition to MAPK cascade signaling, PAMP perception also induces Ca2+ dependent kinases (CDPKs) by regulating Ca2+ influx channels (Ma et al., 2009; Kwaaal et al., 2011). Recent findings indicate that Ca2+ ATPases regulate Ca2+ influx and function to regulate innate immune defenses (Zha et al., 2010). Of particular interest is the Ca2+ ATPase Aca8 which was shown to interact with FLS2, and which may well regulate CDPK signaling through flg22 perception (Fcox et al., 2012).

MPK8 activity has been shown to negatively regulate the expression of OXI1 in order to maintain ROS homeostasis. Remarkably, activation of MPK8 is not limited to the upstream MAPK2 MKK3, as the Ca2+ binding protein calmodulin (CaM) is able to bind and activate MPK8 in an Ca2+-dependent manner (Takahashi et al., 2011). CaM-mediated MPK8 activation is interesting because it bypasses the traditional, sequential activation of MAPKs and also unequivocally links MAPK activation with the ROS burst and ion flux during stress signaling. In addition, CaM also mediates MAPK downregulation. MAP kinase phosphatase 1 (MKP1), which interacts with MPK3, MPK4, and MPK6 (Lim et al., 2002), binds CaM in a Ca2+-dependent manner and stimulates MKP1 phosphatase activity (Lee et al., 2008). The associations between CDPKs and MAPK cascades have recently been review elsewhere (Warzinger et al., 2011).

Much progress has been made in understanding how MAPK signaling functions in plant immunity. In Arabidopsis, 3 of the 60 identified MAP3Ks are involved in defense, namely MEK1 (Asai et al., 2002), EDKI (Freye et al., 2001), and MKK10 (del Pozo et al., 2004; Run et al., 2008). In addition, at least 6 of the 10 identified MAP2Ks (MKK1, MKK2, MKK4, MKK5, MKK7, and MKK9) are involved in defense signaling (Asai et al., 2002; Dsamet et al., 2007; Doci et al., 2007; Zhang et al., 2007b; You et al., 2008). This situation requires tight regulation of the spatial and temporal kinase activities in order to impose specificity upon downstream signaling. To shed light on this regulation, high-throughput methods such as those used by Popescu et al. (2009) are particularly valuable and help to outline MAPK signaling cascades. While this progress may be lauded, further work needs to focus on identifying direct, in vivo kinase substrates and their respective phosphorylation sites. This may bring us closer to bridging the apparent gap between PRRs and MAPK cascades, and to understanding how specificity is achieved among MAPK pathways both spatially and temporally.

ACKNOWLEDGMENTS
This work was supported by grants to John Mundy from the Danish Research Council for Technology and Production (23-03-0076) and the Strategic Research Council (09-061748) and to Milena Roux from the Natural Science Council (11-116368).

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Rasmussen et al. MAP kinase cascades in immunity


Conflicts of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 May 2012; paper pending: 24 May 2012; accepted: 09 July 2012; published online: 24 July 2012.


This article was submitted to Frontiers in Plant Proteomics, a specialty of Frontiers in Plant Science.

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**mRNA decay in plant immunity**

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**Abstract**

Strict regulation of mRNA metabolism is required in plant immunity in order to control defense responses and regulation of immune receptors. Transcription is specific and is typically regulated through activation and release of transcription factors, equally important is transcript degradation. The mechanisms of mRNA decay are well studied, but the mechanisms regarding specificity is are not well understood. Mutants defective in both nonsense mediated decay and decapping display autoimmune phenotypes implying functions in immune regulation. In this review, we will focus on the few published examples concerning mRNA decay and immune responses in *Arabidopsis*.

**Introduction**

mRNA metabolism is strictly regulated to control the fate of cells in response to developmental and environmental stimuli. mRNA metabolism refers to any event in the lifecycle of mRNAs including transcription, processing and degradation. Much is known about changes in transcript levels during plant development (Becker and Theißen, 2003) and in response to abiotic stress (Akhtar et al., 2012; Rasmussen et al., 2013) or biotic stresses including microbial perception (Andreasson et al., 2005; Mao et al., 2011; Qiu et al., 2008). Changes in specific transcript levels are often attributed to changes in their transcription rates, although there are relatively few reports of global or specific transcription rates (Sidaway-Lee et al., 2014). While transcription of genes responding to specific stimuli clearly affects the accumulation of mRNAs (Akhtar et al., 2012), the contribution of mRNA decay is less studied. A primary example of the importance of mRNA decay is illustrated by Xu and Chua (Xu and Chua, 2012, 1) who showed that phosphorylation of Decapping 1 (DCP1) by MAP kinase 6 (MPK6) upon dehydration stress promotes decapping, and that plants expressing a non-phophorylatable version of DCP1 were hypersensitive to osmotic stress.

Eukaryotic mRNAs contain two integral stability determinants, the 5’ 7-methylguanosine triphosphate cap (m7G) and the 3’ poly(A) tail, both of which are incorporated co-transcriptionally. These structures interact with cellular proteins: eukaryotic initiation factor 4E (eIF4E) binds directly
to the 5’ cap and the poly(A) binding protein (PABP) binds to the 3’ poly(A) tail. The binding of eIF4E and PABP promotes translational initiation and, at the same time, prevents degradation by exoribonucleases. Thus, to initiate mRNA decay these determinants must either be removed or the mRNA must be cleaved by an endonuclease followed by an exoribonuclease attack on the newly exposed 3’ and 5’ ends. Eukaryotic mRNAs are primarily degraded by removal of the poly(A) tail with subsequent removal of the 5’ cap and 5’ to 3’ degradation by exoribonucleases, or via 3’ to 5’ degradation by the exosome complex. The components of the Arabidopsis exosome complex are reviewed by Lange and Gagliardi (Lange and Gagliardi, 2010), and Chiba and Green (Chiba and Green, 2009) provide a comprehensive comparison of yeast and plant mRNA decay machinery. Transcripts can also be degraded via nonsense mediated decay (NMD), a eukaryotic RNA surveillance mechanism that controls transcripts with aberrant features and targets them for degradation. These aberrant features include premature termination codons (PTC), PTC-independent long 3’ untranslated regions, 3’ UTR-positioned introns, and upstream open reading frames (Wachter and Hartmann, 2014). In addition to controlling mRNA integrity, NMD has also been described in mammalian cells to target different mRNA isoforms, providing an additional regulation point for important biological processes (Smith and Baker, 2015). This review mainly focuses on mRNA degradation in the context of immune signaling in plants. While most published work in this field concerns microRNA (miRNA) mediated decay of transcripts, we primarily focus here on the few examples of non-miRNA immune mediated mRNA.

Plant innate immunity

Plants have two layers of immunity (Jones and Dangl, 2006). The first layer is comprised of pattern recognition receptors (PRRs) in the plasma membrane, which recognize pathogen-associated molecular patterns (PAMPs). This recognition induces PAMP triggered immunity (PTI) (Greeff et al., 2012). PAMPs and their cognate PPRs include bacterial flagellin (and its peptide derivative flg22) recognized by the receptor FLS2 (Felix et al., 1999; Gómez-Gómez and Boller, 2000), bacterial elongation factor EF-Tu by EFR1 (Kunze et al., 2004; Zipfel et al., 2006) and fungal chitin recognized by CERK1 (Miya et al., 2007). Immune responses triggered by PAMP recognition include protein phosphorylation, induction of defense genes, extracellular alkalization and production of reactive oxygen species (Rasmussen et al., 2012). The second layer of defense employs cytoplasmic immune receptors known as resistance (R) proteins that can detect pathogenic virulence determinants, commonly referred to as effectors, which are injected into the host cell. R proteins recognize
effectors by direct interaction or via effector modification of host proteins and activate effector triggered immunity (ETI) (Jones and Dangl, 2006). PTI and ETI signaling is somewhat similar although ETI responses are prolonged and more pronounced and can include localized cell death (Cui et al., 2014). Most R-proteins are nucleotide-binding domain, leucine-rich repeat (NB-NLR) proteins that can be further separated into coiled-coil- (CC-) or Toll/interleukin-1 receptor- (TIR-) NB-LRRs (Caplan et al., 2008). The mechanism(s) by which R-proteins confer resistance against specific pathogens remain largely unknown, and their signaling mechanisms are probably diverse (Bonardi and Dangl, 2012). Nonetheless, different genes are required for NB-LRR mediated resistance as ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) and PHYTOALEXINDEFICIENT4 (PAD4) are required for TIR-NB-LRR defenses whereas NO DISEASE RESPONSES1 (NDR1) is required for most defense responses mediated by CC-NB-LRRs (Century et al., 1997; Parker et al., 1996). R proteins are tightly regulated as their overexpression may lead to increased immune signaling and autoimmune phenotypes in non-infected plants (Alcázar and Parker, 2011; Nandety et al., 2013). Conversely, plants with loss-of-function R genes are more susceptible to specific pathogen attacks.

**mRNA decay and autoimmunity**

Some mutants implicated in mRNA decay exhibit autoimmune phenotypes. These include protein associated with topoisomerase protein 1 (pat1) involved in decapping of transcripts, as well as upframeshift1 (upf1), upf3 and suppressor with morphogenic effect on genitalia7 (smg7) functioning in non-sense mediated decay (NMD) (Fig. 1A) (Gloggnitzer et al., 2014; Jeong et al., 2011; Rayson et al., 2012a; Riehs-Kearnan et al., 2012; Roux et al., 2015). The autoimmune phenotypes of these NMD deficient mutants resemble those of lesion mimic mutants such as mpk4 (Petersen et al., 2000; Zhang et al., 2012, 2) and accelerated cell death11 (acd11) (Brodersen et al., 2002; Palma et al., 2010, 11) in which autoimmunity is caused by inappropriate activation of ETI. Similar to R protein mediated ETI, autoimmunity in NMD deficient mutants is also repressed at elevated temperatures or high humidity (Alcázar and Parker, 2011; Riehs-Kearnan et al., 2012; Zhu et al., 2010). In addition, inactivation of PAD4 but not NDR1 fully suppresses the retarded growth observed in the smg7 and upf1 NMD deficient mutants. This indicates activation of ETI by one or more TIR-NB-LRRs in these mutants (Riehs-Kearnan et al., 2012).
NMD is apparently regulated during immune responses because plants challenged with the virulent bacteria *Pseudomonas syringae* pv. tomato (*pto*) DC3000 have reduced levels of *UPF1* and *UPF3* transcripts and accumulate canonical NMD target transcripts (Jeong et al., 2011). RNA-seq data have shown that 39 TIR-NB-LRRs and 11 CC-NB-LRRs are upregulated in the *smg7 pad4* double mutant compared to wild type (Gloggnitzer et al., 2014). Interestingly, 19 of these TIR-NB-LRRs
contain putative NMD target features while these features are absent in the 11 upregulated CC-NB-LRRs. The half-lives of all but one of the upregulated TIR-NB-LRR transcripts are increased in the smg7 pad4 double mutant, whereas the half-lives of the upregulated CC-NB-LRR transcripts are unaltered. This indicates that only TIR-NB-LRR transcripts are affected by mutations in SMG7 (Gloggnitzer et al., 2014). Although NMD seems to regulate TIR-NB-LRR transcripts post-transcriptionally and hence regulate ETI, NMD is downregulated after PAMP treatment with flg22. This supports a role for NMD in regulating certain PTI responses (Gloggnitzer et al., 2014). This raises the question of whether the phenotype of NMD deficient mutants is due to an intrinsic mechanism stabilizing NMD targeted defense transcripts such as those of TIR-NB-LRRs.

Alternatively, since a natural variant of the TIR-NB-LRR RPS6 can suppress the autoimmune smg7 phenotype (Gloggnitzer et al., 2014) this NMD deficient phenotype could also be due to activation of RPS6 guarding putative effector targeted steps in the NMD pathway mimicked by mutations in SMG7. Two models explaining autoimmunity in smg7 is illustrated in Figure 2B.

Thus, autoimmunity in mRNA decay mutants may arise from inappropriate activation of R proteins caused by mutations in favored effector targets. An example of this comes from analysis of the decapping mutant pat1 (Roux et al., 2015). Decapping or removal of the mRNA 5’ cap structure is important for exoribonuclease mediated transcript degradation. Mutants impaired in decapping such as dcp1, dcp2 and varicose (vcs) are all seedling lethal and accumulate high levels of capped transcripts (Goeres et al., 2007; Xu et al., 2006, 1). Recent studies have shown that transcript accumulation due to improper decapping or lack of sequential decay by exoribonucleases or the exosome results in induced endogenous gene silencing through increased siRNA levels (Martínez de Alba et al., 2015; Zhang et al., 2015). In addition to gene silencing, miRNA-guided translational repression also seems to be elevated in plant decapping mutants, as also reported for mammalian systems (Brodersen et al., 2008; Eulalio et al., 2007). mRNA decapping occurs in discrete cytoplasmic foci known as processing bodies and, upon activation of PTI with flg22, these foci increase in number (Balagopal and Parker, 2009; Parker and Sheth, 2007; Roux et al., 2015). PAT1 localizes to processing bodies where it functions as a decapping activator (Marnef and Standart, 2010). Arabidopsis PAT1 interacts and is phosphorylated by MPK4, and PAT1 phosphorylation increases after flg22 treatment (Roux et al., 2015). MPK4 functions in transducing signals from PPRs (Rodriguez et al., 2010) and plants lacking MPK4 activity display activation of ETI dependent on the R protein SUMM2 which guards the MPK4 pathway (Petersen et al., 2000, 4; Zhang et al., 2012, 4).

Mutants lacking PAT1 display constitutive defense responses and, similarly to mpk4 mutants, pat1 is immunosuppressed when crossed into a summ2 background. In addition, PAT1 interacts or is in
complex with SUMM2. It is therefore possible that PAT1 is an effector target with a specific function in immunity that is under surveillance by SUMM2 (Roux et al., 2015). Arabidopsis MPK6, another stress related MAP kinase, phosphorylates DCP1 upon dehydration stress and thereby represses decapping (Xu and Chua, 2012, 1). Taken together, these observations indicate that both biotic and abiotic stress signaling can directly influence mRNA decapping.

An example of how mRNA decay may control important steps in a defense pathway includes the plant hormone ethylene that regulates both developmental processes and responses to biotic and abiotic stresses (Johnson and Ecker, 1998). Ethylene is perceived in Arabidopsis by a family of 4 receptors that function as negative regulators (Chang and Stadler, 2001). In the absence of ethylene, these receptors activate Constitutively Triple Response (CTR1), a MAPK kinase kinase (MAP3K) which also functions as a negative regulator of ethylene signaling (Kieber et al., 1993). Ethylene Insensitive 2 (EIN2) is genetically downstream of CTR1 and is a positive regulator promoting ethylene responses through the transcription factors EIN3 and EIN3-like proteins (Alonso et al., 1999, 2; Chao et al., 1997; Guo and Ecker, 2003). In the presence of ethylene EIN3 levels increase rapidly, while in the absence of ethylene EIN3 is down-regulated post-translationally through a ubiquitin/proteasome pathway mediated by the EIN3 binding F-box proteins 1 and 2 (EBF1&2 (Gagne et al., 2004; Guo and Ecker, 2003, 3; Potuschak et al., 2003; Yanagisawa et al., 2003)). Both EBF1 and EBF2 transcripts have been found to be targets of the Exoribonuclease 4 (XRN4) and hence accumulate in xrn4 mutants which results in reduced levels of EIN3 protein (Olmedo et al., 2006; Potuschak et al., 2006, 4). Tight regulation of EIN3, likely through EBF1 and EBF2, is essential for fine-tuning immunity as EIN3 positively regulates transcription of the PRR FLS2 by binding to its promoter (Boutrot et al., 2010). EIN3 also regulates transcription of Isochorismate Synthase 1 (ICS1 also known as SID2), involved in the biosynthesis of the defense hormone salicylic acid. In contrast to its effects on FLS2, EIN3 represses transcription of ICS1 and thereby inhibits salicylic acid accumulation (Chen et al., 2009). How and why EIN3 reciprocally regulates two genes important for mounting proper immune responses remains a bit of a mystery. Nonetheless, it seems clear that EIN3 itself is regulated via EBF1 and EBF2 mRNA decay (Olmedo et al., 2006; Potuschak et al., 2006). Expression of Ethylene Response Factor 1 (ERF1) is directly induced by EIN3 (Solano et al., 1998). ERF1 links ethylene and jasmonate signaling in Arabidopsis for appropriate expression of defense responses against pathogens (Lorenzo et al., 2003). As for EBF1 and EBF2, ERF1 transcripts are also targeted by XRN4 and hence accumulate in xrn4 mutants (Nguyen et al., 2015). XRN4 is homologous to yeast XRN1 which degrades RNA species with a single phosphate group at the 5’ end (Kastenmayer and Green, 2000; Souret et al., 2004). It is therefore likely that the ERF1 transcripts,
as well as those of EBF1 and EBF2, that accumulate in xrn4 mutants are uncapped and that decapping in fact regulates these transcripts prior to XRN4 mediated decay. This has not yet been tested experimentally.

In addition to the above examples, miRNAs provide an additional control over mRNA decay and a direct link to regulation of immunity. Post transcriptional gene silencing (PTGS) is a key regulatory mechanism in controlling immune responses in plants (Seo et al., 2013). In PTGS, double stranded RNAs are cleaved into 20-24 nucleotide small RNA species by DICER-like enzymes. One of the two RNA strands then associates with ARGONAUTE (AGO) proteins which are catalytic components of the RNA-induced silencing complex (RISC) promoting mRNA cleavage (Meister, 2013). Different classes of small RNA species including micro RNAs (miRNAs) and short interfering RNAs (siRNAs) are involved in PTGS. miRNAs are transcribed from the genome by RNA polymerase II and, due to regions of reverse sequence complementarity, primary or pri-miRNAs form hairpin-like secondary structures (Ha and Kim, 2014). In plants, mature miRNAs are formed in the nucleus by cleavage of pri-miRNAs by DICER-LIKE1 (DCL1) proteins in complex with SERRATE and HYL1. Mature miRNAs are then exported to the cytosol where they associate with AGO1 and promote mRNA cleavage (Ha and Kim, 2014). miRNAs has been shown to function in plant immunity (Li et al., 2010; Navarro et al., 2006). In response to PAMP perception, miRNA miR393 accumulates and negatively regulates auxin signaling by targeting transcripts encoding the auxin receptors TIR1 (transport inhibitor 1), AFB2 (auxin signaling F-box protein 2) and AFB3 (Navarro et al., 2006). Arabidopsis encodes a number auxin response factors (ARFs) that can promote or repress the transcription of genes containing auxin responsive elements (Hagen and Guilfoyle, 2002). The auxin-regulated Aux/IAA proteins sequester ARFs and thereby repress auxin signaling (Liscum and Reed, 2002). TIR1 is part of a ubiquitin-ligase complex interacting with Aux/IAA proteins and marking them for degradation (Gray et al., 2001). Therefore, as a consequence of PAMP triggered miR393 accumulation, Aux/IAA proteins are stabilized resulting in repressed auxin signaling (Navarro et al., 2006). Overexpression of transgenic miR393 increases resistance to pto DC3000, while overexpression of AFB1 (a paralog to TIR1 and AFB2/3 that is resistant to miR393 mediated degradation) renders plants more susceptible (Navarro et al., 2006). This supports a function of miRNA in regulating proper responses during PTI. Virulent bacterial pathogens have evolved effector proteins that can suppress the miRNA pathway and thereby repress host immunity. During infection, Pto DC3000 can employ the effector protein AvrPtoB that represses transcription of miR393a and miR393b which both encode miR393 (Navarro et al., 2008). In addition, the effector protein AvrPto stabilizes miR393 precursors thereby reducing
the levels of mature miR393 and rendering plants more susceptible to infection (Navarro et al., 2008). Since AvrPto and AvrPtoB both interact with and inhibit PRRs including FLS2 (Dodds and Rathjen, 2010; Göhre et al., 2008; Xiang et al., 2008) it is possible that these effectors do not only target the miRNA pathway, but also suppress PTI signaling downstream of FLS2 and other PRRs. Other miRNAs including miR160 and miR167 are also upregulated during PTI, and both target members of the ARF transcription factor family. This further supports the involvement of miRNA mediated gene silencing in auxin signaling during PTI (Fahlgren et al., 2007).

Transcripts encoding R protein immune receptors have also been shown to be direct targets of miRNAs. miR472 negatively regulates several CC-NB-LRRs by targeting conserved regions encoding ATP binding p-loop domains (Boccara et al., 2014; Lu et al., 2006). It is likely that in the absence of infection miR472 promotes degradation of CC-NB-LRR transcripts and that activation of PTI down regulates or pacifies miR472 to permit accumulation of CC-NB-LRR transcripts resulting in increased resistance (Boccara et al., 2014). Consistent with this, miR472 deficient and over-expressor plants are respectively more resistant and susceptible to pto DC3000 (Boccara et al., 2014). Similarly, tomato miR482 targets a group of CC-NB-LRRs and results in the production of secondary trans-acting siRNAs that target other immune regulators. Such secondary targets include PEN3 involved in basal immunity and a proteasomal subunit that is also likely to function in resistance (Shivaprasad et al., 2012). In addition, and as seen for Arabidopsis miR472 and miR393, tomato miR482 mediated silencing is impaired in infected plants, likely due to a bacterial effector. Tomato plants infiltrated with an hrcC mutant of Pto. DC3000 which is incapable of delivering effectors into host plants showed impaired accumulation of miR482 target transcripts compared to plants infiltrated with virulent Pto. DC3000 (Shivaprasad et al., 2012). This effector mediated up-regulation of resistance gene expression via the suppression of host RNA silencing might have evolved as a counter defense against pathogens similar to ETI.

Plant miRNAs represent a fraction of the total pool of small RNAs which includes siRNAs and secondary siRNAs (Meyers et al., 2008). In contrast to miRNAs, siRNAs usually perform auto silencing, while trans-acting siRNAs target mRNAs transcribed from other loci. This amplifies the effects of siRNA and miRNA mediated PTGS (Ghildiyal and Zamore, 2009). For example, the Arabidopsis RPP5 locus encodes seven TIR-NB-LRR proteins including SNC1 and RPP4. Most of these R gene paralogs are all repressed by 21-nucleotide siRNAs corresponding to their LRR regions which are likely generated from antisense transcription from SNC1 (Yi and Richards, 2007).
Conclusion

Our current knowledge of immune regulated mRNA decay and its specificity remains limited. Control of R proteins through miRNA-mediated mRNA decay implies direct roles in regulating appropriate immune responses. The examples described above also suggest that the general decay pathways comprising the exosome, exoribonucleases and NMD are important for immune signaling as well as for responses and adaptations to abiotic stress. It is difficult to identify the target transcript specificities of these pathways, in contrast to the sequence homology based identification of miRNA-mediated decay targets. Nonetheless, since mutations in several of the key components in mRNA decay pathways have autoimmune phenotypes, they are themselves likely targets of pathogen effectors. This issue needs further investigation to ensure that conclusions regarding the functions of these decay components are not based on potential pleiotropic phenotypes of their loss-of-function mutants, but rather reflect their true in vivo functions.

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The mRNA decay factor PAT1 functions in a pathway including MAP kinase 4 and immune receptor SUMM2

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Abstract

Multi-layered defense responses are activated in plants upon recognition of invading pathogens. Transmembrane receptors recognize conserved pathogen-associated molecular patterns (PAMPs) and activate MAP kinase cascades, which regulate changes in gene expression to produce appropriate immune responses. For example, *Arabidopsis* MAP kinase 4 (MPK4) regulates the expression of a subset of defense genes via at least one WRKY transcription factor. We report here that MPK1 functions in complexes *in vivo* with PAT1, a component of the mRNA decapping machinery. PAT1 is also phosphorylated by MPK4 and, upon flagellin PAMP treatment, PAT1 accumulates and localizes to cytoplasmic processing (P) bodies which are sites for mRNA decay. Pat1 mutants exhibit dwarfism and de-repressed immunity dependent on the immune receptor SUMM2. Since mRNA decapping is a critical step in mRNA turnover, linking MPK4 to mRNA decay via PAT1 provides another mechanism by which MPK4 may rapidly instigate immune responses.

Keywords decapping; immunity; MAP kinases; mRNA decay; phosphorylation

Subject Categories Microbiology, Virology & Host Pathogen Interaction; Plant Biology; RNA Biology

DOI 10.15252/embj.201488645 | Received 2 April 2014 | Revised 2 December 2014 | Accepted 11 December 2014

Introduction

Plant innate immunity employs multilayered defense responses comprised of two overlapping mechanisms. In the first layer, plant pattern recognition receptors detect invading microorganisms by the presence of conserved pathogen-associated molecular patterns (PAMPs) (Boller & Felix, 2009). PAMP recognition is exemplified by the binding of the bacterial flagellin-derived flg22 peptide to the leucine-rich repeat-receptor-like kinase flagellin sensing 2 (FLS2) (Gomez-Gomez et al., 2001; Chinchilla et al., 2006). PAMP recognition initiates downstream signaling, including production of reactive oxygen species, calcium influx, MAP kinase activation and global changes in gene expression that induce PAMP-triggered immunity (PTI) (Chisholm et al., 2006; Zipfel, 2009). Adapted pathogens have evolved effector proteins that are delivered into host cells to compromise PTI by evading PAMP detection or suppressing defense responses. In the second layer of immunity, plant resistance (R) proteins have evolved to directly or indirectly recognize the activities of pathogen effectors (Jones & Dangl, 2006). In the best-studied examples, R proteins are found to guard host proteins or complexes (guardes) manipulated by specific pathogen effectors. Pathogen detection via R proteins leads to induction of strong defenses and to a form of host programmed cell death known as the hypersensitive response (HR) to sequester infections. These responses are collectively termed effector-triggered immunity (ETI) (Jones & Dangl, 2006).

Changes in phosphorylation are important regulatory mechanisms in cellular signaling. Activation of mitogen-activated protein (MAP) kinases occurs within 10 min of PAMP application (Asai et al., 2002; Boller & Felix, 2009) and relies on sequential phosphorylations between MAPKK-kinases (MEKK), MAPK kinases (MKK) and MAP kinases (MPK) (Pitzschke et al., 2009; Andreesson & Ellis, 2010; Rasmussen et al., 2012). In *Arabidopsis*, several MPKs are activated by PAMPs. A cascade comprising MEKK1-MKK4/5-MPK3/6 was initially found to be involved in PTI downstream of FLS2 (Asai et al., 2002; Droillard et al., 2004; Gao et al., 2008). Similarly, flg22 treatment activates another cascade including MEKK1, MKK1/2 and MPK4 (Petersen et al., 2000; Ichimura et al., 2006; Mészáros et al., 2006; Suarez-Rodriguez et al., 2007; Gao et al., 2008; Qiu et al., 2008a).
Interestingly, Flg22-induced activation of MPK3, MPK4, and MPK6 is dependent on MKK1, while MPK3 and MPK6 are also activated by MKK4 (Mészáros et al., 2006). Thus, FLS2 activates two cascades, one with an unknown MEKK and MKK4/5-MPK3/6, the other with MEKK1-MKK1/2-MPK4. More recently, the closest homologue of MPK4, MPK11, was shown to also be activated by PAMPs (Betheke et al., 2012).

**mpk4** mutants were originally found to exhibit autoimmunity, and MPK4 thus appeared to function genetically as a negative regulator of defense responses (Petersen et al., 2000; Droillard et al., 2004). However, MPK4 is activated in response to pathogens and PAMP elicitation (Droillard et al., 2004; Teige et al., 2004; Ichimura et al., 2006; Brader et al., 2007; Qi et al., 2008a) which is counter-intuitive for a negative regulator. Subsequently, it was shown that activated MPK4 interacts with and phosphorylates MAP kinase substrate 1 (MKS1), bringing about the release of the transcription factor WRKY33 and induction of the expression of the PHYTOLALEXIN DEFICIENT 3 (PAD3) gene required for biosynthesis of the antimicrobial camalexin (Andreasson et al., 2005; Qi et al., 2008b). This illustrates how MPK4 functions as a positive regulator of PTI. Since it was recently reported that MPK4 is a target for manipulation by pathogen effectors (Zhang et al., 2007, 2012), and as **mpk4** mutant phenotypes are partially suppressed by mutations in the R protein SUMM2 (suppressor or **mpk4** mkk2) (Zhang et al., 2012), one model is that MPK4 is a PTI guerdie whose absence triggers ETI.

Apart from regulation by transcription factors, mRNA translation and degradation also regulate gene expression, especially when they are reprogrammed to stabilize bulk mRNAs and favor mRNAs required for an appropriate stress response (Jiao et al., 2010; Brodersen et al., 2008; Xu & Chua, 2009, 2011; Motomura et al., 2012) as well as the exoribonuclease XRN4 (Olmedo et al., 2006; Potuschak et al., 2006; Gregory et al., 2008; Rymarquis et al., 2011; Vogel et al., 2011). It is thought that DCP1, DHH1 and DCP5 form mRNPs for translational repression of target mRNA, which are then subject to decapping by recruitment of DCP2 and VCS and digestion by XRN4 (Xu & Chua, 2009, 2011). Importantly, although **PAT1** functions have not previously been studied in plants, three homologues are encoded in the *Arabidopsis* genome, each of which contains a conserved C-terminal domain. The decapping activator Sm-like (LSM) proteins, which interact with **PAT1** in eukaryotes (Salgado-Garrido et al., 1999; Bonnerot et al., 2000; Bouveret et al., 2000; Tharun et al., 2000; Tharun, 2009), have recently been characterized in *Arabidopsis* (Perea-Resa et al., 2012; Golisz et al., 2013). It was found that LSM1-7 proteins form a complex and *lsm1* mutants accumulate capped mRNA. Furthermore, VCS, DHH1 and **PAT1** homologs were identified in LSM1 immunoprecipitates (Golisz et al., 2013).

The decapping complex plays important roles in eukaryotic development. In contrast, links between mRNA decapping and stress signaling are just being uncovered (Jiao et al., 2010; Buchan et al., 2011; Munchel et al., 2011; Park et al., 2012), and how decapping may be involved in regulating immune systems is largely unknown. Here, we characterize the *Arabidopsis* homologue of the mRNA decay regulator **PAT1**. We show that **PAT1** functions in decapping of mRNA. Furthermore, **PAT1** is phosphorylated in response to flg22 and localizes to discrete, punctate foci in the cytosol. **PAT1** also interacts with MPK4 and with the R protein SUMM2 in planta, and the absence of **PAT1** triggers SUMM2 dependent immunity. This indicates that **PAT1** is regulated by MPK4 in a pathway whose disruption leads to ETI via SUMM2.

**Results**

**At** **PAT1** is an Sc** **PAT1** orhologue and interacts with **MPK4 in planta**

We previously conducted a yeast two-hybrid screen to identify *Arabidopsis* proteins that interact with MPK4 (Andreasson et al., 2005). In addition to the MPK4 substrate **MPK1**, we identified two clones encoding **Pat1** (At1g79090), an mRNA decapping stimulator involved in post-transcriptional gene regulation (Coller & Parker, 2005). Two **PAT1** homologs are encoded in the *Arabidopsis* genome (*At** **PAT1**1, *At** **PAT1**2, *At** **PAT1**3). The steady-state expression level of **PAT1** and the homologs compared to the housekeeping gene *ACTIN8* (At1g49240) was analyzed by RT-PCR (Supplementary Fig S1A). We focused on **PAT1** as the other two homologs were not identified by yeast two-hybrids. To confirm the **PAT1**-MPK4 interaction in planta, doubly transgenic *Arabidopsis* lines were generated in the *Ler mpk4-1* background that expressed **PAT1** with a C-terminal Myc tag and HA-tagged MPK4 under the control of their own promoters. Anti-Myc immunoprecipitation from either MPK4-HA or double transgenic MPK4-HA/Pat1-Myc tissue detected a 50 kDa band corresponding to MPK4-HA only in double transgenic lines (Fig 1A). Thus, MPK4 and **PAT1** can be found in complex in *Arabidopsis*. 
Yeast PAT1 engages with translating mRNPs and is involved in translational repression and decapping activation (Marnef & Stan-dart, 2010). Since the function of PAT1 in Arabidopsis was unknown, we examined whether it functions similarly to yeast PAT1. To this end, a full-length Arabidopsis PAT1 cDNA was cloned from Col-0 (Supplementary Fig S1C and D) and transformed into wild-type yeast (B4742) and a yeast mutant (Y15797) in which yeast PAT1 was replaced with a G418 resistance cassette (BY4742 (YCR077c) pat1Δ::KanMX). In contrast to the wild-type, yeast lacking PAT1 (pat1Δ) display a temperature-sensitive phenotype and are impaired at 37°C but grow normally at 30°C (Tharun et al., 2005). This phenotype is reverted to wild-type in yeast containing Arabidopsis PAT1, as growth at 37°C was restored (Fig 1B). As an additional control, we transformed Arabidopsis PAT1 into wild-type

![Figure 1](https://example.com/figure1.png)
yeast (B4742/AtPAT1), and this grew similarly to wild-type at 30°C and almost as well at the wild-type at 37°C (Fig 1B). The expression of Arabidopsis PAT1 in yeast was confirmed by anti-PAT1 immunoblotting of yeast protein extracts (Supplementary Fig S1E). This provides compelling evidence for the orthologous functions of these yeast and Arabidopsis PAT1 proteins. As PAT1 is found in complex with MPK4, these results provide a link between MPK4 and post-transcriptional regulation of mRNA stability.

We next analyzed the interaction between PAT1 and conserved components of mRNA decapping. PAT1-LSM1-7 complexes function in mRNA decapping and deadenylation (Bouveret et al., 2000; Tharun, 2009; Haas et al., 2010; Ozgur et al., 2010; Totoaro et al., 2011). We therefore transiently expressed in Nicotiana benthamiana LSM1-GFP and PAT1-HA and then immunoprecipitated LSM1 with GFP Trap beads. PAT1-HA could be detected in LSM1 immunoprecipitates but did not adhere to GFP Trap beads in the absence of LSM1-GFP (Fig 1C). This is consistent with the detection of peptides corresponding to PAT1 and its homologues in LSM1 immunoprecipitates (Golisz et al., 2013) and supports a role of PAT1 in mRNA decapping. In other organisms, interactions between PAT1 and LSM1 are robust, while those between PAT1 and other mRNA decapping proteins, including the DCP1-DCP2 complex and XRN1, are more transient (Bouveret et al., 2000; Nissan et al., 2010; Ozgur et al., 2010). This is consistent with our difficulty in detecting DCP1 in complex with PAT1 in Arabidopsis (Supplementary Fig S1F).

### PAT1 is required for decapping of selected mRNAs

In order to determine whether PAT1 behaves as an activator of mRNA decapping, we used 5' RACE to compare the levels of capped mRNAs in Col-0 and pat1-1 mutants. To this end, we identified an allele, pat1-1 (Salk_040660), with a T-DNA insertion in the last exon of PAT1 (Supplementary Fig S1C). We also generated an anti-PAT1 antibody against a C-terminal peptide (Supplementary Fig S1D). Immunoblotting of Col-0 protein extracts with this antibody detected a clear band around 90 kDa. In contrast, no protein could be detected in pat1-1 mutant extracts (Supplementary Fig S1G). This indicates that pat1-1 harbors either a truncated version of PAT1, no PAT1 protein, or levels of the protein that are below detection.

5' RACE was performed on transcripts known to be degraded by the decapping complex (EXPL1; UGT87A2) (Perea-Resa et al., 2012), as well as a housekeeping transcript EIF4A1. We found that capped EXPL1 and UGT87A2 accumulated in pat1-1 mutants, while capped EIF4A1 mRNA was present in equal amounts in Col-0 and pat1-1 (Fig 1D). This indicates that PAT1 plays a role in mRNA decay via decapping.

### PAT1 is an MPK4 substrate

Since MPK4 and PAT1 are found in complexes in planta, we asked whether PAT1 is an MPK substrate. PAT1 contains 5 Ser-Pro (SP) motifs which are commonly phosphorylated by MPks (Pearson et al., 2001; Ubersax & Ferrell, 2007) (Supplementary Fig S1D). To characterize PAT1 phosphorylation in vivo, we identified PAT1 phosphopeptides by mass spectrometry. Since MPK3/4/6 are activated by flagellin and by virulent strains of Pseudomonas syringae pv. tomato (Pto) DC3000 (Asai et al., 2002; Brader et al., 2007; Suarez-Rodriguez et al., 2007; Bethke et al., 2009, 2012; Rasmussen et al., 2012), PAT1 was immunoprecipitated from extracts of untreated control and flg22-treated wild-type Col-0 or PAT1-GFP transgenic lines. Bands corresponding to PAT1-GFP (130 kDa) were excised from the gel (Supplementary Fig S2), subjected to in-gel tryptic digestion, and peptides were extracted. Phosphopeptides were enriched by TiO₂ chromatography and analyzed by liquid chromatography (RP-HPLC) coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). This identified several phosphopeptides from PAT1-GFP IPs that were not detectable in the negative control (Col-0) (Table 1; Supplementary Fig S2). The most abundant phosphopeptide, based on the extracted ion chromatogram from the LC-MS/MS analysis, revealed phosphorylation of Ser208 in an SP motif (Fig 2A). Another peptide was identified with phosphorylation of Ser343. However, this site is not within an SP motif, making it a less likely site for phosphorylation by MPks. Importantly, both these peptides have previously been detected in Arabidopsis by mass spectrometry (Phosphat Database, http://phosphat.mpimp-golm.mpg.de/). It should be noted that the PAT1 phosphopeptides were detected both whether or not the sample had been treated with flg22. Thus, under the conditions used here, PAT1 was phosphorylated and remained so after exposure to flg22.

To determine whether PAT1 is a substrate of a specific MPK, we carried out in vitro kinase assays using immunoprecipitated, PAMP-activated MPKs with purified His₅-PAT1 protein. Phosphorylated His₅-PAT1 was detectable as a radioactive band around 95 kDa after incubation with flg22-activated MPK4 (Fig 2B), while MPK6 caused only low levels of PAT1 phosphorylation and MPK3 did not significantly alter PAT1 phosphorylation (Supplementary Fig S3). Each MPK was also incubated with the generic MPK substrate myelin basic protein (MBP) to verify their activation (Fig 2B; Supplementary Fig S3). This confirms that activated MPK4, and to a lesser extent, MPK6, is responsible for the phosphorylation of PAT1. A version of His₅-PAT1 with Ser208 mutated to alanine (S208A) was also incubated with MPK4 and MPK6 IPs, and this mutant

### Table 1. Phosphopeptides identified in PAT1-GFP IPs by mass spectrometry analysis.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Phospho-sites</th>
<th>m/z</th>
<th>Mascot score</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSFVSYPPGCSIPDPQQR</td>
<td>1 (S208)</td>
<td>950.93005</td>
<td>71</td>
</tr>
<tr>
<td>SSFVSYPPGCSIPDPQQR</td>
<td>2 (S208, S200)</td>
<td>990.91333</td>
<td>46</td>
</tr>
<tr>
<td>SSGNYDGLMGFCDLR</td>
<td>1 (S343)</td>
<td>929.87323</td>
<td>70</td>
</tr>
<tr>
<td>SSGNYDGLMGFCDLR</td>
<td>2 (S343, S342)</td>
<td>969.85663</td>
<td>39</td>
</tr>
</tbody>
</table>

*aPhosphosites refers to the number and in brackets the location of the phosphorylation detected by MS analysis. Potential phosphorylation sites are indicated in bold letters. m/z refers to the mass-to-charge ratio of the tabulated phosphopeptides.
had significantly lower levels of phosphorylation (Fig 2B). This supports the identification of S208 as a key phosphorylation site in

**PAT1.**

**pat1** mutants exhibit autoimmunity similar to **mpk4**

Given that MPK4 is an important immune regulator in *Arabidopsis* and that *mpk4* mutants have de-repressed defense responses, we examined whether **PAT1** may also be involved in immunity. The **pat1-1** mutant has a distinct leaf serration phenotype and a slightly smaller rosette than Col-0 (Fig 3). A similar rosette phenotype was seen in another T-DNA insertion line (**pat1-2**, WiscDsLox_734_D04 in Supplementary Figs S1C and S4), indicating that this phenotype is due to loss-of-function of **PAT1**. Importantly, however, the phenotype of **pat1-1** is not as extreme as **mpk4-2**, which is much smaller and has more pronounced leaf curling and reduced fertility as well as constitutive defense gene expression (Petersen et al., 2000; Fig 3).

In order to determine whether **pat1-1** is a constitutive defense mutant similar to **mpk4**, quantitative reverse-transcription PCR (qRT-PCR) was used to measure the steady-state level of the pathogenesis-related **PR1** and **PR2** genes in adult plants (Fig 4A). Previous work showed that the enhanced defense response of **mpk4** mutants is suppressed by mutations in **EDS1** (Brodersen et al., 2006). Thus, **eds1-2** was crossed with **pat1-1** to explore the **pat1** phenotype in the absence of this regulator (Fig 3). Compared to Col-0, **pat1-1** mutants accumulated 1,000-fold more **PR1** and 150-fold more **PR2** transcripts (Fig 4A). In contrast, the levels of **PR** mRNAs in **pat1-1 eds1-2** double mutants were similar to those in wild-type (Fig 4A). Under the same conditions, **mpk4-2** mutants accumulated 4,000-fold more **PR1** and 800-fold more **PR2** transcripts (Fig 4A).

We found that the elevated **PR** gene expression in **pat1-1** correlated to an enhanced resistance to infection by syringe-infiltrated *Pto* DC3000 when compared to Col-0 (Fig 4B). While *Pto* DC3000 growth reached 5.5 cfu/cm² in Col-0, **pat1-1** mutants supported tenfold lower accumulation of *Pto* DC3000. In this experiment, **eds1-2** mutants showed enhanced bacterial growth as expected for this mutant with compromised defense responses (Fig 4B). **pat1** disease resistance is **EDS1** dependent, as bacterial growth **pat1-1 eds1-2** double mutants was similar to that in **eds1-2** (Fig 4B). These findings indicate that, similar to **mpk4**, **pat1** mutants express **EDS1**-dependent autoimmunity in the absence of microbes.
PAT1 detection in P-bodies is induced by PAMPs

Processing bodies (PBs) are cytoplasmic granules involved in both mRNA decay and translational repression pathways (Kulkarni et al., 2010). PAT1 is a conserved PB component in yeast (Rodriguez-Cousino et al., 1995), C. elegans (Boag et al., 2008), Drosophila (Marnef et al., 2010) and mammals (Scheller et al., 2007) and PBs can be significantly induced in Arabidopsis by hypoxia and heat stress (Weber et al., 2008). To investigate whether PAT1 is found in PBs, we produced transgenic Arabidopsis lines that express PAT1-GFP from its native promoter complementing the pat1 phenotype (Supplementary Fig S5A and B). We detected GFP signal in small numbers of distinct foci by confocal microscopy in roots of young seedlings (Fig 5A). Within 20 min of flg22 treatment, a significant increase in the number of GFP-positive foci could be seen in the root tips (Fig 5A). To test whether these foci correspond to PBs, we treated the roots with cycloheximide in DMSO which is known to abrogate PB formation in plants (Goeres et al., 2007). This revealed that cycloheximide inhibited flg22-induced foci (Fig 5A). Importantly, the control DMSO treatment did not reduce the number of foci (Supplementary Fig S6A and B). As a control, we also tracked the localization of the known decapping component VCS (Xu et al., 2006). Similar to PAT1-GFP, VCS-GFP was seen in flg22-induced PBs and absent when treated with cycloheximide (Fig 5B). Interestingly, PAT1 protein, which is hardly detectable under steady-state conditions, also accumulated in response to flg22 treatment, with a peak by 60 min, and a return to normal levels after 2 h (Fig 5C). PAT1-GFP mirrors this effect and was similarly up-regulated in response to flg22 treatment, as detected by anti-GFP Western blotting of seedling protein (Fig 5D). Importantly, PAT1 transcript levels were not highly induced by flg22 treatment in Col-0 seedlings (Supplementary Fig S6C), suggesting that PAT1 induction occurs post-transcriptionally. These data indicate that activation of PTI leads to up-regulation of PAT1 protein levels by an unknown post-transcriptional mechanism, and this facilitates the detection of PAT1 in PBs. In addition, PTI could induce PB formation as part of cellular reprogramming, and PAT1 may localize to PBs to engage in this process.

The MPK4 suppressor summ2 also suppresses the pat1 resistance phenotype

Autoimmunity caused by loss of different components of the MPK4 kinase cascade can be suppressed by mutations in the resistance protein SUMM2 (Zhang et al., 2012). An explanation for this is that SUMM2 keeps this PAMP responsive pathway under surveillance and that mutations in its components mimic the effects of microbial effectors that prevent phosphorylation within or below the cascade (Zhang et al., 2007). To further probe the connection between MPK4 and PAT1, we generated pat1-1 summ2-8 double mutants. These mutants retained the leaf serration phenotype of pat1-1 single mutants (Supplementary Fig S7A). However, double mutants no longer accumulated excessive PR1 and PR2 transcripts (Fig 6A) and displayed summ2 levels of susceptibility to syringe-infiltrated Pto DC3000 (Fig 6B). Importantly, the pat1 growth phenotype was not caused by overexpression of SUMM2 transcripts, as the level of SUMM2 in pat1-1 was similar to wild-type (Supplementary Fig S7B). To test whether the accumulation of 5’ capped transcripts in pat1-1 (Fig 1D) was merely an effect of inappropriate activation of SUMM2, we tested the accumulation of 5’ capped UGT87A2 in an XRN1 sensitivity assay. XRN1 degrades all uncapped RNA leaving 5’ capped RNA intact (Blewett & Goldstrohm, 2012). The accumulation of 5’ capped versus uncapped UGT87A2 transcripts was at similar levels in pat1-1 and pat1-1 summ2-8 (Fig 6C). Col-0 and summ2-8 plants had similarly reduced 5’ capped versus uncapped ratios but, importantly, these were much lower than the levels of pat1-1 and pat1-1 summ2-8 (Fig 6C). Therefore, the accumulation of capped transcripts in pat1-1 mutants is not an artifact of defense activation but reflects a role for PAT1 in mRNA decay. In order to determine whether the SUMM2-mediated resistance in pat1 mutants is specific to pathogens of a specific class, we compared susceptibility to pathogens with different lifestyles. Pto DC300 is a hemi-biotrophic
pathogen, which grows in living tissue during early infection. In contrast, the necrotrophic fungal pathogen *Botrytis cinerea* relies on dying tissue for its propagation in plants (Glazebrook, 2005). Infection of pat1-1 summ2 double mutants with *B. cinerea* resulted in similar growth of the fungus as detected in summ2-8 single mutants (Supplementary Fig S8).

To more accurately address to what extent PAT1 phosphorylation is MPK4 dependent and to measure PAT1 phosphorylation profiles before and after flg22 treatment, we generated PAT1-HA transgenic lines in the *mkk1/2 summ2-*8 background. The suppression of *mpk4* by summ2-8 is only partial, but the autoimmune phenotype is fully suppressed in *mkk1/2 summ2* triple mutants. We next applied quantitative mass spectrometry (iTRAQ) to PAT1-HA IPs from Col-0 and *mkk1/2 summ2* and this revealed increased phosphorylation stoichiometry of PAT1 at Ser208 in untreated Col-0 compared to *mkk1/2 summ2* plants (Fig 6D). Flg22 treatment leads to increased levels of PAT1 Ser208 in both genotypes, but the levels in Col-0 were higher than what we found for PAT1 in *mkk1/2 summ2* (Fig 6D). Since flg22 treatment augments PAT1 Ser208 phosphorylation in the absence of MKK1/2, MPK4 might be activated by other upstream kinases or PAT1 may be phosphorylated by MPK6. Nevertheless, these data indicate that flg22 treatment leads to increased phosphorylation of PAT1 at Ser208 and the MKK1/2 MPK4 pathway contributes to this phosphorylation.

Our data indicate that PAT1 is part of the pathway including MPK4 and the SUMM2 R protein. SUMM2 and PAT1 might thus interact in planta. To test this, we transiently expressed and immunoprecipitated PAT1-GFP in *N. benthamiana* also transiently expressing MPK4-HA or SUMM2-HA. When PAT1-GFP was pulled down using GFP Trap beads, we could easily detect MPK4-HA and SUMM2-HA in the immunoprecipitates (Fig 7A). To verify the association of MPK4, SUMM2 and LSM1, we also pulled down PAT1-HA and detected MPK4-GFP, SUMM2-GFP and LSM1-GFP in the immunoprecipitates but not Myc-GFP (Supplementary Fig S9). To further confirm the association of PAT1 and SUMM2, we used bimolecular fluorescence complementation (BiFC), which produces a fluorescent readout upon reconstruction of YFP. The BiFC assay showed detectable YFP signal in the cytoplasm when PAT1 and SUMM2 were co-expressed (Fig 7B). No signals were observed when PAT1 was expressed with another R protein, At4g12010, implying that the association/reconstruction is specific (Fig 7B, bottom panel). This indicates that PAT1 is in complex with SUMM2 in planta. Thus, MPK4 and PAT1 are both physically and genetically linked to SUMM2.

**Discussion**

In this study, we identify the *Arabidopsis* decapping component PAT1 and show it to be an interactor and substrate of MPK4 in plant innate immunity. Furthermore, we demonstrate that *Arabidopsis* PAT1 complements yeast *pat1*Δ mutants, indicating that the function of this conserved protein is maintained in plants. The accumulation of capped mRNAs in pat1 mutants is consistent with a role for PAT1 in the mRNA decapping pathway.

**Pat1 mutants exhibit a distinct leaf serration phenotype** (Supplementary Fig S4) resembling those of miRNA-loss-of-function mutants (Nikovics et al, 2006) such as *abh1-8* (Gregory et al, 2008), *serrate* (Grigg et al, 2005) and hypomorphic alleles of *ago1* (Morel, 2002). This suggests that PAT1 may have a role connected to microRNA activity. *Arabidopsis* decapping mutants such as *dcp1*, *dcp2* and *vcs* accumulate lower levels of certain miRNAs (Motomura et al, 2012). Mutants with a *pat1*-like phenotype, such as *vcs*, *su6* and *amp1*, have revealed new components in miRNA-mediated translational repression (Brodersen et al, 2008; Yang et al, 2012; Li et al, 2013). In *Drosophila*, HpAt interacts with components of the miRNA machinery including AGO1 and GW182 (Barišić-Jäger et al, 2013). The connection between the mRNA decay activator HpAt and the miRNA effector complex may provide a link to promote the transition of mRNA from translation to degradation. Although PAT1 may regulate targets of miRNA-mediated translational repression, the mechanism by which translational repression occurs in *Arabidopsis* is still under investigation.
We also find here that PAT1 is an MPK4 substrate and that pat1 mutants exhibit autoimmunity as does mpk4. The connection between MPK4 and PAT1 is further supported by suppression of the pat1 constitutive defense phenotype by loss-of-function of the SUMM2 R protein (Fig 6). However, SUMM2 deficiency only partially rescues mpk4 mutants (Zhang et al, 2012), thus it is
possible that this partial rescue represents a SUMM2 PAT1 branch in the MPK4 pathway. Nevertheless, the pat1 constitutive defense phenotype is suppressed by summ2 such that pat1 summ2 mutants display a wild-type phenotype in response to biotrophic and necrotrophic pathogens (Fig 6; Supplementary Fig S8). Since PAT1 and its homologues in tomato, DCN, and X. laevis may function redundantly during PTI. An alternative explanation is that we simply have not yet tested a pathogen whose infection strategy could reveal a role of PAT1 in immunity.

mRNA decapping is not the only mRNA regulatory pathway characterized by constitutive defense responses. Indeed, mutants of nonsense-mediated decay including upf3-1, upf1-5 and smg7 mutants display autoimmune phenotypes (Jeong et al., 2011; Rayson et al., 2012a; Riehs-Kearnan et al., 2012; Shi et al., 2012). Their phenotypes are also suppressed by mutations in immune regulators such as eds1 and pad4 (Rayson et al., 2012b; Riehs-Kearnan et al., 2012), similar to what we find for pat1. This suggests that these components could also be under surveillance and may be suppressed by mutations in specific R genes. Recently, it was suggested that nonsense-mediated decay controls turnover of R gene mRNAs and cause autoimmunity in smg7 (Gloggnitzer et al., 2014). However, since autoimmunity in smg7 depends on a specific allele of the R protein RPS6, it is also possible that SMG7 is under surveillance. Most significantly, this suggests that plants have developed complex sensors to monitor the integrity of these pathways, which is consistent with the importance of differential gene expression in response to pathogen perception.

We detected PAT1 phosphorylation by PAMP-activated MPK4, and weakly also by MPK6 in vitro. We also identified PAT1 Ser208 and Ser343 as in planta phosphorylation sites by mass spectrometry. Since PAT1 phosphorylation was reduced in vitro when Ser208 was mutated to Ala, this site may be a key target of MPK4 (Fig 2B). Interestingly, Ser208 is conserved in Phycomyces patens (moss), rice, the Arabidopsis PAT1 homologues, and Xenopus PATL2, but not in human or yeast PAT1 (Supplementary Fig S10). Although Ser208 corresponds to an SP site in Xenopus PATL1/2, PATL1
is known to be phosphorylated on Ser62 by an unknown kinase (Marnef et al., 2010). Thus, Ser208 may represent a plant-specific site or mechanism. While Ser208 is conserved in plant PAT1 orthologs, Ser342/3 is not. As Ser342/3 does not correspond to SP sites, they may be phosphorylated by kinases other than MPKs. Furthermore, we detected PAT1 phosphorylation in planta irrespective of PAMP treatment.

PAT1 phosphorylation by MPKs has not been shown in any system, although several phosphosites have been identified in human and yeast PAT1 proteins (Phosphoelm, http://phospho.elm.eu.org/ and Phosida http://www.phosida.com/databases). However, other decapping complex members are subject to stress-induced MPK-mediated phosphorylation. For example, human DCP1a is phosphorylated by c-Jun N-terminal kinase in response to stress (Rzeczkowski et al., 2011). Similarly, Ste20 phosphorylates yeast DCP2 upon glucose deprivation (Yoon et al., 2010). In both cases, phosphorylation seems only to be required for P-body formation and not for general decapping (Yoon et al., 2010; Rzeczkowski et al., 2011). Arabidopsis MPK6 was shown to specifically phosphorylate DCP1 in plants during dehydration stress (Xu & Chua, 2012). Thus, the regulation of mRNA decay machinery by MPKs during stress responses seems to be a key mechanism in plants and other organisms, although exactly how this affects mRNA turnover remains elusive.

**Materials and Methods**

**Plant materials and growth conditions**

Arabidopsis thaliana ecotype Columbia (Col-0) was used as a control. Seeds for T-DNA insertion lines were from NASC (Nottingham, UK).
The T-DNA lines for At1g79090 (PAT1), both of which have insertions in the last exon, were Salk_040660 (here named pat1-1) and WiscDsLox437D04 (pat1-2). The T-DNA insertion in At1g12280 (SUMM2) summ2-8 (SAIL_1152A06), mkk1/2 (Zhang et al., 2012) and edsl-2 (Parker et al., 1996) have been described. Genotyping primers for newly described T-DNA lines are provided in Supplementary Table S1. Arabidopsis plants were grown in 9 × 9 cm pots at 22°C with a 8-h photoperiod, or on plates containing Murashige-Skoog (MS) salts medium (Duchefa), 1% sucrose and 1% agar with a 16-h photoperiod.

Cloning and transgenic lines

The genomic PAT1 (At1g79090) DNA sequence (without stop codon), plus 2 kb upstream from the start codon, was amplified from Col-0 genomic DNA and cloned into pENTR-D-TOPO (Invitrogen). The entry clone was recombined into pGWB513, pGWB517 and pGWB504 (Nakagawa et al., 2007) to obtain a C-terminal HA, Myc and GFP tags, respectively. The expression clones were transformed into Agrobacterium tumefaciens strain GV3101 and transformed into Col-0 plants by floral dipping. Transformants were selected on hygromycin (30 µg/ml) MS agar and survivors tested for protein expression by Western blotting.

The VCS promoter region was amplified and inserted into pGEM-T easy and next into the HindIII Sall in pBnGFP to generate pBnPvCVC-GFP. The VCS coding region was amplified and cloned into pGEM-T easy next inserted into Sall and Kpnl in pBnPvCVC-GFP and transformed into Agrobacterium LBA4404 and transformed into Col-0 plants by floral dipping. The cDNA of LSM1 (At3g14080) without stop codon was cloned into pENTR-D-TOPO (Invitrogen) and recombined into 35S promoter-containing pGWB505 (Nakagawa et al., 2007) to obtain C-terminal GFP-tagged LSM1. Genomic DNA of MPK4 and cDNA of SUMM2 were cloned into pENTR-D-TOPO and recombined into pGWB514 (Nakagawa et al., 2007) to obtain C-terminal HA-tagged constructs. The genomic DNA and promoter of DCPI was cloned into pENTR-D-TOPO and recombined into pGWB513 to obtain C-terminal HA-tagged constructs under the control of the native promoter. Clones were transformed into A. tumefaciens strain GV3101 and used for transient expression in N. benthamiana or floral dipping in Arabidopsis. Genomic PAT1-GFP was transformed into the Col-0 background. DCPI-HA was dipped into the Col-0 background and T3 progeny were crossed with Col-0/PAT1-GFP T4 lines to obtain double transgenic lines. Genomic PAT1-Myc was transformed into Ler mpk4-1/MPK4-HA transgenic lines (described in Petersen et al., 2000) to obtain double transgenic lines.

The entry clones of PAT1 and SUMM2 were further recombined into pcYGW and pnYGW (Hino et al., 2011), respectively, obtaining the N- and C-terminal split YFP tags used for BiFC. A4tg12010 clones were obtained by the same procedure. Clones were transformed into A. tumefaciens strain GV3101 and used for transient expression in N. benthamiana.

Mutagenesis of His6-PAT1

Site-directed mutagenesis of Ser208 to Ala was accomplished using pET15b-PAT1 as a template for PCR mutagenesis. The primers used were PAT1 S208A F&R (see Supplementary Table S1).

PAT1 protein purification and in vitro kinase assays

For in vitro experiments, PAT1 protein was purified from E. coli. The PAT1 Cl DNA was cloned into pET15b (for an N-terminal His fusion) and transformed into E. coli BL21 (pLysS). Protein expression was induced by overnight treatment with 0.5 mM IPTG at 18°C, added to cells at OD600 = 0.6. PAT1 protein was insoluble and thus purified from inclusion bodies using BugBuster (Novagen). Proteins were solubilized in 6 M urea, 0.7% N-lauroylsarcosine, 100 mM Tris-HCl pH 8 and refolded overnight at 4°C in 0.88 M L-arginine, 55 mM Tris-HCl, 2 mM NaCl, 0.88 mM KCl, protease inhibitors. Protein was then dialyzed against 20 mM Tris-HCl pH 8, 100 mM NaCl using 3,500 MWCO dialysis tubing. Purified protein was concentrated using Centriprep 30K spin columns (Millipore) and then diluted with glycerol to a final concentration of 0.1 mg/ml.

For kinase assays, Col-0 plants were immersed in 200 nM flg22 for 10 min of to activate MAP kinases. MPK3, 4 and 6 were immunoprecipitated from 3 mg total protein extracted from flg22-treated tissue using 2 µg of each of their specific antibodies (Sigma) and 30 µl EZView protein A agarose beads (Sigma). Each microgram of purified myelin basic protein (MBP, Sigma) or 20 µg His6-PAT1 protein was incubated with washed MPK immunoprecipitates for 60 min at 37°C with 3 µCi γ-ATP in kinase buffer (62.5 µM ATP, 100 mM Tris pH 7.5, 150 mM NaCl, 150 mM MgCl2, 10 mM EGTA, 5 mM DTT, Phosstop inhibitor (Roche)). Kinase reactions were diluted with 4× SDS buffer and boiled for 5 min before loading on 12% SDS-PAGE gels. Following electrophoresis, gels were stained with Coomassie Brilliant Blue and incubated with gel drying buffer, followed by drying on a Bio-Rad gel dryer. Dried gels were exposed to a phosphor screen overnight.

Yeast transformation

Yeast strains pat1Δ (BY4742 YCR077c) pat1Δ::KanMX and B4742 were obtained from Euroscarf. PAT1 was cloned from A. thaliana Col-0 Cl DNA into pENTR-D-TOPO and recombined into yeast expression vector pVV215 (C-terminal HA tag, -URA selection; (Van Mullem et al., 2003) by Gateway recombination. Yeast pat1Δ and B4742 cells were transformed using lithium acetate/polyethylene glycol according to the Clontech Yeast Protocols handbook. Transformed yeast was selected on SD-URA agar plates and re-streaked after 3–4 days onto fresh selection plates. Pat1Δ and wild-type-transformed yeast was grown in liquid culture overnight, and protein was extracted from pelleted cells by vortexing with glass beads in 1× SDS extraction buffer. Boiled proteins were subjected to SDS-PAGE and anti-PAT1 immunoblotting. For temperature sensitivity assays, overnight cultures of wild-type, pat1Δ and PAT1-expressing transformants were plated on YPD and grown at 30 and 37°C for 3 days.

Flg22 kinetics

Seedlings were grown on MS agar (Col-0) or MS agar containing 30 µg/ml hygromycin (Col-0/PAT1-GFP) for 5 days before being transferred to MS liquid medium in 24-well plates. After 10 days, seedlings were treated by the addition of flg22 to a final concentration of 100 nM (2 seedlings per well × 3 wells per treatment time
point). Seedlings were harvested at the indicated times and immediately frozen in liquid nitrogen for later RNA or protein extraction.

**Semi-quantitative and qRT-PCR**

Total RNA was extracted from seedlings with TRI Reagent (Sigma). RNA samples were treated with DNase Turbo DNA-free (Ambion), quantified with a NanoDrop spectrophotometer (Thermo Scientific) and reverse-transcribed into cDNA with SuperScript III reverse transcriptase (Invitrogen). For semi-quantitative reverse-transcription PCR (RT-PCR), Col-0 seedling cDNA was used as a template for PCR with primers specific for ACTIN8, PAT1, PATI1H1 or PATI1H2 using Sigma Jumpstart REDTaq Readymix. PCR products (20 μl) were separated on 2% (w/v) agarose gels and visualized with ethidium bromide. Brilliant II SybrGreen master mix (Agilent) was used for qPCR analysis, normalized to UBQ10 expression and plotted relative to Col-0 expression level. These experiments were repeated in three independent biological replicates, each with three technical replicates, and representative data are shown. Standard error is represented by error bars on figures, and statistical significance is indicated by letters above error bars. These are derived from one-way ANOVA with Tukey’s multiple comparison test (GraphPad Prism).

**RNA extraction and RACE PCR**

RNA extraction used TRI reagent and 10 μg RNA was used for S‘ RACE according to instructions (First Choice RACE, Ambion). PCR was carried out on 1 μl of products from reverse transcription of capped RNA using DreamTaq polymerase (Fermentas) with 25 cycles. RACE PCR products (10 μl) were separated on 2% (w/v) agarose gels and visualized with ethidium bromide.

**Quantification of capped versus uncapped transcripts**

Total RNA was extracted from seedlings with NucleoSpin RNA columns (Machery-Nagel). To remove RNA with no S’ cap structure, 1 μg of total RNA was incubated with 1 unit XRN1 (New England Biolabs) or no enzyme at 37°C for 1 h. Next RNA was reverse-transcribed into cDNA with SuperScript III reverse transcriptase (Invitrogen). UGT87A2 transcript accumulation was measured by qPCR using SybrGreen master mix (Agilent) and normalized to ACT2. Calculating S’ capped versus uncapped transcripts was done by comparing transcript levels from XR1N1 and mock-treated samples for the individual genotypes.

**Confocal microscopy**

Col-0/PAT1-GFP plants were grown on MS agar containing 30 mg/ml hygromycin for 5 days. Seedlings were placed on glass microscope slides with water, 1% DMSO or 100 nM flg22 for 20 min. For following cycloheximide and DMSO treatment, seedlings were removed from flg22 and placed on new glass microscope slides. Cycloheximide was used at 100 μg/ml in a 1% DMSO dilution, and the control was done with 1% DMSO. Imaging was done using a Leica SP5 inverted microscope.

### Infection assays

**Pseudomonas syringae pv. tomato DC3000** (Pto DC3000) strains were grown in overnight culture in Kings B medium supplemented with appropriate antibiotics. Cells were harvested by centrifugation and pellets resuspended in sterile water to OD_{600}=0.002. Bacteria were infiltrated with a needleless 1-mm syringe into four leaves on four plants per genotype and maintained in growth chambers for 3 days. Samples were taken using a cork-borer (6.5 mm) to cut leaf disks from four leaves per plant and four plants per genotype. Leaf disks were ground in water, serially diluted and plated on Kings B with appropriate selection. Plates were incubated at 28°C and colonies counted 2–3 days later. These experiments were repeated in three independent biological replicates, and representative data are shown.

For drop inoculation of *B. cinerea* (strain B05.10), 10 μl of 2.5 × 10^5 spores/ml in Gamborg B5/2% sucrose (pH 5.6) was placed on the adaxial surface of fully expanded leaves of 4-week-old plants and sampled by harvesting 10–15 leaf disks per genotype at each time point, namely 0, 2 and 3 days. Data are the average of 3 biological replicates. Disease severity was measured as accumulation of the *B. cinerea* CUTINASE A transcript by qPCR relative to *A. thaliana* α-SHAGGY KINASE (At5g26751) (primers are according to Gachon & Saindrenan, 2004).

### Transient expression in *Nicotiana benthamiana*

*Agrobacterium tumefaciens* strains were grown in LB medium supplemented with appropriate antibiotics overnight. Cultures were spun down and resuspended in 10 mM MgCl₂ to OD_{600}=0.8. *A. tumefaciens* strains carrying PAT1-GFP and MPK4-HA or SUMM2-HA were mixed 1:1 and syringe-infiltrated into 3-week-old *N. benthamiana* leaves. Samples for protein extraction were harvested 2 days post-infiltration (dpi). *Agrobacterium tumefaciens* strains carrying either PAT1-HA, LSM1-GFP or PAT1-HA + LSM1-GFP were mixed 1:1 and syringe-infiltrated into 3-week-old *N. benthamiana* leaves. Samples for protein extraction were harvested 3 dpi. For BiFC, *A. tumefaciens* strains carrying PAT1 fused to the N-terminal part of YFP and SUMM2 or At4g12010 fused to the C-terminal part were mixed 1:1 and syringe-infiltrated into *N. benthamiana* leaves at an final OD_{600}=0.8. Confocal microscopy on leaf disks was conducted 2 days post-infiltration under a Leica SP5 inverted microscope.

### Protein extraction and immunoprecipitation in *Nicotiana benthamiana*

Leaves were ground in liquid nitrogen and extraction buffer [50 mM Tris-HCl pH 7.5; 150 mM NaCl; 10% (v/v) glycerol; 10 mM DTT; 10 mM EDTA; 1% (v/v) PVP; protease inhibitor cocktail (Roche); 0.1% (v/v) IGEPAL CA-630 (Sigma); Phosstop (Roche) added at 2 ml/g tissue powder. Samples were clarified by 20 min centrifugation at 4°C and 13,000 rpm. Supernatants (1.5 ml) were adjusted to 2 mg/ml protein and incubated 2 h at 4°C with 20 μl GFPTrap-A beads (Chromotek) or anti-HA antibodies (2 μg, Santa Cruz) and 30 μl EZview protein A agarose beads (Sigma). Following incubation, beads were washed four times with IP buffer, before adding 30 μl 2× SDS and heating at 95°C for 5 min.
Arabidopsis protein extraction and immunoprecipitation for mass spectrometry analysis

Leaves from adult plants were ground in liquid nitrogen and extraction buffer (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 10% (v/v) glycerol; 5 mM DTT; 2 mM EDTA; protease inhibitor cocktail (Roche); 0.1% (v/v) IGEPAL CA-630 (Sigma) and Phosstop (Roche) added at 2 ml/g tissue powder. Samples were clarified by 20 min centrifugation at 4°C, 13,000 rpm. Supernatants (45 ml) were adjusted to 3 mg/ml protein and incubated 4 h at 4°C with 50 µl GFPTrap-A beads (Chromotek) or anti-HA antibodies (4 µg, Santa Cruz) and 100 µl EZview protein A agarose beads (Sigma). Following incubation, beads were washed four times with IP buffer before adding 2× SDS and heating to 95°C for 5 min.

SDS-PAGE and immunoblotting

SDS-PAGE gels were prepared with either 8, 10 or 12% cross-linking. Proteins were loaded and gels run at 100–150 V for 1.5 h before electroblotting onto PVDF membrane (GE Healthcare). Membranes were rinsed in TBS and blocked for 1 h in 5% (w/v) non-fat milk in TBS-Tween (0.1% (v/v)). Antibodies were diluted in blocking solution to 10 ng/l in 2 ml/g tissue powder. Samples were clarified by 20 min centrifugation at 4°C, 13,000 rpm. Supernatants (45 ml) were adjusted to 3 mg/ml protein and incubated 4 h at 4°C with 50 µl GFPTrap-A beads (Chromotek) or anti-HA antibodies (4 µg, Santa Cruz) and 100 µl EZview protein A agarose beads (Sigma). Following incubation, beads were washed four times with IP buffer before adding 2× SDS and heating to 95°C for 5 min.

Antibodies

Polyclonal anti-PAT1 antibodies were generated by immunizing rabbits with synthetic peptides derived from the N-terminus [EQRIPDRTKLYPEPQ] and C-terminus [KRSMLGSQKTEPVLS] of PAT1. Antibodies (final bleed) were affinity-purified against the C-terminal peptide (Eurogentec). Antibody specificity was verified by immunoblotting with plant extracts derived from Col-0 and pat1-1 tissue. Mouse anti-HA and anti-GFP antibodies were obtained from Santa Cruz. Rabbit anti-GFP antibodies were obtained from AMS Biotechnology. Anti-MPK3, anti-MPK-4 and anti-MPK-6 antibodies were obtained from Sigma. Secondary antibodies were obtained from Promega.

In-gel digestion, TiO₂ chromatography and mass spectrometry

Bands excised from SDS-PAGE were chopped into small pieces and incubated for 1 h in 50 mM TEAB, acetonitrile (50:50) on a shaker at room temperature. After incubation, the sample was centrifuged shortly and the supernatant was discarded. Gel pieces were dried in a vacuum centrifuge for 15 min and subsequently 30 µl of trypsin (10 ng/µl) in 20 mM TEAB pH 7.5 was added to cover the dried gel pieces. The solution was incubated in 4°C for 30 min. After incubation, the trypsin solution was replaced with 30 µl 20 mM TEAB pH 7.5 and the tube was incubated at 37°C overnight. Peptides from the digestion solution after incubation were recovered in a low binding Eppendorf tube (Sorenson Bioscience), and the gel pieces washed with 50% acetonitrile for 15 min to extract more peptides. The washing solution was mixed with the recovered peptides, and the peptide solution was lyophilized. Phosphopeptides were purified by titanium dioxide (TiO₂) chromatography (Larsen et al., 2005). Lyophilized peptides were redisolved in loading buffer for TiO₂ chromatography (80% acetonitrile, 5% TFA, 1 M glycolic acid), and 0.3 mg TiO₂ beads (GL Science, Japan) were added to the solution and incubated for 10 min. After incubation, the beads were pelleted by centrifugation and the supernatant was removed. The beads were washed once with 80% acetonitrile, 1% TFA and once with 10% acetonitrile, 0.1% TFA. Phosphopeptides were eluted using 1% ammonium hydroxide and desalted and concentrated prior to LC-MS/MS using a Poros Oligo R3 micro-column (Engholm-Keller et al., 2012).

For quantitative phosphopeptide analysis, the tryptic peptide mixtures after in-gel digestion were labeled with iTRAQ 4 plex (according to the manufacturer’s protocol) and the samples were mixed prior to TiO₂ enrichment as described above. The non-phosphorylated peptides were concentrated and desalted using a Poros Oligo R3 micro-column (Engholm-Keller et al., 2012). The non-phosphorylated peptides were used to normalize the PAT1 protein level.

LC-MS/MS analysis was performed using an EASY-LC system (Proxeon, Thermo Fisher Scientific) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) as described previously (Engholm-Keller et al., 2012). Peptides were separated using a 20 min gradient from 0–34% B-buffer (A-buffer: 0.1% formic acid; B-buffer: 90% acetonitrile, 0.1% formic acid). The data-dependent analysis was performed using one MS full scan in the area 300–1,800 Da performed in the Orbitrap with 60,000 in resolution, followed by the five most intense ions selected for MSMS (collision induced dissociation) performed in the linear ion trap.

Raw data from the LTQ-Orbitrap-XL were processed using the Proteome Discoverer (PD) program (Thermo Fisher Scientific, Bremen, Germany). The data were searched against the Arabidopsis database (33,596 sequences; 13,487,687 residues) using the Mascot 2.2 version database. The parameters for the database search were as follows: Enzyme—trypsin; missed cleavages—2; MS mass accuracy—10 ppm; MSMS mass accuracy—0.8 Da; Variable modifications—Phosphorylation (S, T, Y), Oxidation (met), Propionamide (C). The identified peptides were filtered for 1% false discovery rate using the ‘Fixed Value PSM Validator’ in PD. All identified phosphopeptides were manually validated. For iTRAQ-labeled phosphopeptides, the PD quantitation node was used and the data were normalized based on the non-phosphorylated peptides.

Supplementary information for this article is available online: http://emboj.embopress.org

Acknowledgements

We thank YueLin Zhang for providing summ2-8 and mkk1/2 summ2-8 seeds. This work was supported by the Danish Research Council for independent Research, Natural Sciences Grant #10-084139 (M.P.) & Postdoctoral Fellowship
mRNA decapping component PAT1 in immunity

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#11-116368 (M.E.R.) & Technology and Production Sciences #11-106302. KP was supported by an EMBO Long-Term Fellowship and a Marie Curie International Incoming Fellowship. We thank Sukawai Vonvisutikun for technical help and Dr. Tsuyoshi Nakagawa (Shimane University) for providing Gateway binary pGWB vectors. All confocal work was done at Center for Advanced Bioimaging. MRL was supported by the Lundbeck Foundation (Junior Group Leader Fellowship).

Author contributions
MER, MWR, KP, JM and MP conceived and designed the experiments. MER, MWR KP, SL, MRL, JC, AMR, LS, WZ and GB performed experiments. MER, MWR, JM and MP analyzed the data. MER, MWR, JM and MP wrote the manuscript.

Conflict of interest
The authors declare that they have no conflicts of interest.

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Figure S1: Pat1 alleles, protein and immunoblotting

Phylogenetic tree of PAT1 orthologues (neighbor-joining tree generated from MAFFT multiple alignment in Geneious version 5.6.6 created by Biomatters (http://www.geneious.com/). AtPAT1, Arabidopsis thaliana; CePAT1, C. elegans; OsPAT1, Oryza sativa; HPat, Drosophila melanogaster; HsPAT1, Homo sapiens; ScPAT1, Saccharomyces cerevisiae; XlPAT1, Xenopus laevis. RT-PCR analysis of PAT1, ACTIN8, PAT1H1 and PAT1H2 gene expression in Arabidopsis Col-0 seedlings. C. PAT1 gene structure. Promoter (grey box), exons (white boxes) and introns (black lines). Start codon is marked ATG. T-DNA insertion sites of pat1-1 (Salk_040660) and pat1-2 (WiscDs_437D04) are marked with black triangles in exon. D. PAT1 contains N-terminal, Pro-rich, middle and Pat-C domains. Peptides for antibody generation are indicated by black pentagons. Potential SP phosphosites are marked with black stars. Amino acid numbers are above each domain. E. Anti-PAT1 immunoblotting of yeast protein extracts from pat1∆ transformed with AtPAT1 and untransformed pat1∆. AtPAT1 corresponds to the 89 kDa band seen in the pat1∆ transformed with AtPAT1. A nonspecific band indicated by *.
F. Co-IP between PAT1-GFP and DCP1-HA. Transgenic lines expressing DCP1-HA (1,2) or PAT1-GFP and DCP1-HA (3,4) were treated with water (-) or 200nM flg22 (+) for 15 mins. Proteins were extracted and subjected to HA and GFP IPs. Immunoblots of inputs (20 ug each, top panel), GFP IPs (middle panel) and HA IPs (bottom panel) were probed with anti-GFP and anti-HA antibodies. GFP IPs were also probed with anti-MPK4 antibodies as a control for PAT1-GFP IPs. Molecular weights indicated on the right in kDa.

G. The anti-PAT1 antibody does not cross-react with any protein in pat1-1 mutant total protein extracts. Protein extracts (35 µg) derived from wild-type Col-0 and pat1-1 probed with 1:250 dilution of anti-PAT1 and alkaline phosphatase-conjugated anti-rabbit antibodies.

Figure S2: Mass spectrometry analysis of PAT1-GFP. Colloidal Coomassie-stained SDS-PAGE of GFP IPs from Col-0 or Col-0/PAT1-GFP treated with water or flg22. Molecular weight in kDa indicated on the left.

Figure S3: MPK3 does not phosphorylate PAT1 in vitro. MPK3 was immunoprecipitated from Col-0 seedlings treated with water (-) or 200 nM flg22 (+) for 10 minutes. MPK3 IPs were incubated with His6-PAT1 or MBP for 30 mins at 37°C before SDS-PAGE. Autoradiogram (top panel), Coomassie-stained gel for loading control (bottom). Molecular weight in kDa indicated at right.
Figure S4: *Pat1* mutants have a leaf serration phenotype. 4-week-old (left) or 8-week-old (right) plants grown under short-day conditions.
Figure S5: PAT1-GFP complements the pat1-1 phenotype.

A. Plants photographed at 5-weeks of growth in short-day conditions where PAT1-GFP complements the pat1-1 leaf serration phenotype. B. The accumulation of PR1 and UGT87A2 transcripts in pat1-1 are reverted when introducing PAT1-GFP under control of the native PAT1 promoter. Transcript levels were quantified by qRT-PCR using RNA extracted from 5-week-old plants grown in soil under short-day conditions. Fold change is represented relative to Col-0 and normalized to ACT2. Standard error of the mean is indicated by error bars (n=3) and statistical significance determined by ANOVA followed by Tukey's test is shown as letters above error bars.
Figure S6: effect of flg22 treatment on PAT1 transcript and PAT1-GFP protein.
Confocal microscopy with root elongation zones of five-days-old Col-0/PAT1-GFP seedlings. Roots treated in DMSO show no significant change in localization or amounts of GFP signal. The number of GFP foci in PAT1-GFP seedlings is clearly induced upon treatment with 1μM flg22 on glass slides for 20 min (second panel). No reduction in GFP foci is seen after treatment with DMSO for 60 min (bottom panel). The scale bar corresponds to 10 μm. qRT-PCR analysis of PAT1 transcripts in Col-0 seedlings treated with time course of 100 nM flg22 with time in minutes post-treatment as indicated. UBQ10 was used for normalization. Expression is displayed as fold change (where Col-0 at t=0 mins is 1). Standard error of the mean is indicated by error bars (n=3).
Figure S7: *pat1 summ2* mutant phenotype
A. 4-week-old plants grown under short-day conditions.
B. qPCR analysis of *SUMM2* transcripts in 4-week-old *Arabidopsis Col-0, pat1-1, summ2-8* and *pat1-1 summ2-8* plants. *UBQ10* was used for normalization. Expression is displayed as fold change (where Col-0 = 1). Standard error of the mean is indicated by error bars (n=3).

Figure S8: Infection of *pat1-1 summ2-8* mutants with necrotrophic fungus *B. cinerea*. Drop inoculation infection of 4-week-old *Col-0, pat1-1, summ2-8* and *pat1-1 summ2-8* plants sampled at 0, 2 and 3 days. Data are the average of 3 biological replicates. Disease severity was measured as accumulation of the *B. cinerea* *CUTINASE A* transcript by qPCR relative to *A. thaliana* α-SHAGGY KINASE (*At5g26751*).
Figure S9: PAT1 is associated with LSM1, MPK4 and SUMM2 in N. benthamiana. Proteins were transiently co-expressed (PAT1-HA with LSM1-GFP, MycGFP, MPK4-GFP or SUMM2-GFP) in N. benthamiana and tissue and harvested 2 days post-infiltration. Anti-HA immunoblot of PAT1-HA IPs are shown in the top panel. Anti-GFP immunoblot of PAT1-HA IPs are shown in the middle panels. The bottom panel show the presence of LSM1-GFP, Myc-GFP and MPK4-GFP in the input. Although we were unable to detect SUMM2-GFP in the input SUMM2-GFP was easily detected in the enriched PAT1-HA IP. Molecular weights are shown in kDa at left.
Figure S10: Multiple alignments of key regions of PAT1 homologues from several kingdoms. Alignment used MAFFFT algorithm in Geneious version 5.6.6 created by Biomatters (http://www.geneious.com/). Pink boxes mark predicted SP sites; yellow boxes mark phosphorylation sites identified by MS in this study.
AOC3 is an MPK4 substrate and its overexpression induce immunity against *pst* DC3000

**Introduction**

In response to pathogen perception plants rapidly instigate local defense mechanisms. Two systems for detecting pathogens are: (i) Pathogen recognition receptors on the plant cell surface that recognize conserved pathogenic structures termed pathogen associated molecular patterns (PAMPs) and (ii) cytoplasmic immune receptors that recognize either injected pathogen effectors or modifications of effector host targets (Rasmussen et al., 2012). Both mechanisms induce rapid localized responses but can also induce systemic acquired resistance mediated by phytohormones (Robert-Seilaniantz et al., 2011). The transduction of and/or responses to defense signaling in *Arabidopsis* involves at least three phytohormones that mediate appropriate responses toward to specific classes of pathogens. These hormones are salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) or their derivatives (Bari and Jones, 2008). In response to pathogen perception, plants produce these hormones in ratios depending on the specific class of pathogen (Robert-Seilaniantz et al., 2011). Plants usually respond to biotrophic pathogens feeding on living plant tissue by producing SA, whereas necrotrophic pathogen feeding on dead tissue typically induces production of JA and ET (Glazebrook, 2005). The lifestyle of many pathogens are not easily classified as either biotrophic or necrotrophic. Therefore, the otherwise antagonistic SA and JA/ET pathways are likely correlated to tailor response to specific pathogens (Adie et al., 2007). Some biotrophic plant pathogens have developed ways of suppressing SA mediated defenses by inducing the antagonistic JA signaling pathway. For example, this is accomplished by introducing the bacterial JA analog coronatine into the plant cell leading to suppression of defense responses against biotrophic pathogens (Cui et al., 2005; Laurie-Berry et al., 2006). Although coronatine in some instances mimics JA, this is not always true. For example, plants respond to coronatine by opening their stomata allowing pathogens to enter the apoplastic space while JA induces stomatal closure (Melotto et al., 2006; Suhita et al., 2004).
The synthesis of defense related hormones including JA is upregulated in response to recognition of specific pathogens. The first steps in the biosynthesis of JA occur in the chloroplast (Fig. 1). Here α-linolenic acid present in the thylakoid membrane is oxygenated by a lipoxygenase (LOX) to yield the fatty acid peroxide 13(S)-HpOTrE. This peroxide is next dehydrated by ALLENE OXIDE SYNTEHASE (AOS) to the unstable epoxide 12,13(S)-epoxyoctadecatrienoic acid which is further modified by ALLENE OXIDE CYCLASE (AOC) to produce 9S,13S-12-oxo-phytodienoic acid (OPDA) (Hamberg and Fahlstadius, 1990; Schaller et al., 2004; Vick and Zimmerman, 1979; Zimmerman and Feng, 1978). In aqueous solutions the epoxide produced from AOS degrades rapidly (half-life of less than 30 seconds at 0°C) into mainly α- and γ-ketols while a minor fraction undergoes cyclization producing a racemic mixture of OPDA (Hamberg, 1988; Ziegler et al., 1999). This is in contrast to the AOC catalyzed conversion which produces only the optically pure enantiomer 9S,13S-OPDA used in downstream JA biosynthesis (Hamberg and Fahlstadius, 1990).

Due to the short half-life of the AOS product, it is likely that the functions of AOS and AOC are coupled in a complex, although no direct evidence of such an interaction has been shown. Translocation of OPDA to the peroxisomes, where the remaining steps in the pathway occur, is not fully understood. OPDA itself can function as a signaling compound and therefore its translocation across the chloroplast envelope is likely to be tightly regulated (Dave and Graham, 2012). Translocation from the cytosol to the peroxisome is actively facilitated by ABC transporters (Gerhardt, 1983; Theodoulou et al., 2005). In the peroxisomes OPDA is reduced enzymatically by an OPDA reductase (OPR) and subsequently undergoes three rounds of β-oxidation resulting in the production of JA (Fig. 1) (Li et al., 2005; Schaller et al., 2000).
In the chloroplasts α-linolenic acid is oxygenated by LOX to generate 13(S)-HpOTrE which is further modified by AOS to yield the unstable epoxide 12,13(S)-epoxyoctadecatrienoic acid. In the absence of AOC the epoxide rapidly hydrolyzes into mainly α- and γ-ketols while a minor fraction undergoes cyclization into a racemic mixture of OPDA. In contrast, AOC catalyzed cyclization of the epoxide produces only the optically pure 9S,13S-OPDA which then is transported out of the chloroplast to the cytosol by an unknown mechanism. From the cytosol 9S,13S-OPDA is actively transported into the peroxisomes by an ABC transporter where it is reduced by OPR and undergoes three rounds of β-oxidation resulting in the production of JA.

Figure 1: The JA biosynthesis pathway.
AOC displays a greater substrate specificity than AOS and it thus appears that AOC confers additional specificity on the JA biosynthetic pathway (Schaller et al., 2008). The Arabidopsis genome contain four genes encoding AOC enzymes: AOC1 (At3g25770), AOC2 (At3g25780), AOC3 (At3g25760) and AOC4 (At1g13280). These four genes share great sequence identity (Fig. 2) and are most likely functionally redundant to each other as individual T-DNA loss-of-function mutants do not exhibit JA-related phenotypes (Stenzel et al., 2012). Promoter analysis using a GUS reporter system has also shown partially redundant promoter activities during development: (i) In fully developed leaves AOC1, AOC2 and AOC3 promoter activity are apparent throughout all leaf tissue, whereas AOC4 promoter activity are specific to vascular tissue; (ii) only AOC3 and AOC4 promoter activities are detected in roots; (iii) AOC1 and AOC4 promoter activities are detected during flower development (Stenzel et al., 2012). In Arabidopsis AOC2 is the best characterized of the four AOC enzymes and its crystal structure confirms previous findings that AOC forms a functional trimer (Hofmann et al., 2006). The regulation of the JA biosynthesis pathway is not fully understood, although positive feedback regulation seems able to induce accumulation of transcripts encoding genes involved in the biosynthetic pathway of JA (Wasternack and Hause, 2013). Interestingly all four AOC enzymes can perform both homo and heteromerization in vivo with each other. This might serve as a level of regulation in JA biosynthesis since some spatial and temporal promoter activities are observed for the different AOC promoters (Stenzel et al., 2012).

Figure 2: Alignment of AOC1-4 from Arabidopsis
Peptide sequences were aligned with “CLC Main Workbench” without their chloroplast transit peptides (first 81-84 aa). Putative MAP kinase phosphorylation sites (SP/AP) are indicated with red background coloring. Secondary structures according the crystal structure of AOC2 are indicated above the alignment.
One of the early responses to PAMP perception is activation of MAP Kinase (MPK) signaling (Rasmussen et al., 2012). MPK4 activation is linked to recognition of the bacterial PAMP flagellin and thus functions in transducing immune signaling through phosphorylation of downstream targets (Qiu et al., 2008; Rodriguez et al., 2010; Roux et al., 2015). MPK4 is a target for the bacterial effector protein AvrB indicating MPK4s pivotal role in immune signaling (Cui et al., 2010). This is confirmed by the fact that SUPPRESSOR OF MKK1 MKK2 2 (SUMM2), a cytoplasmic immune receptor, is activated in the absence of MPK4 or MPK4 activity (Zhang et al., 2012). In investigating the molecular function of MPK4, a yeast two-hybrid screen was initiated searching for MPK4 interacting proteins (Andreasson et al., 2005). AOC3 was identified in this screen as a full-length cDNA clone. Here we characterize the interaction between AOC3 and MPK4. We show that AOC3 is a substrate of MPK4 and that overexpressing AOC3 in Arabidopsis trigger enhanced immunity. We also provide evidence that MPK4 is associated with the chloroplast, that may indicate that MPK4 can regulate AOC3 activity/stability inside the chloroplast in response to pathogens.

**Results**

**AOC3 Interacts with MPK4 in planta.**

In a previously conducted yeast two-hybrid screen we identified AOC3 as an interactor of MPK4. To confirm the AOC3-MPK4 interaction in planta, we transiently expressed in Nicotiana benthamiana AOC3-GFP along with MPK4-HA and then immunoprecipitated AOC3-GFP. MPK4-HA was detected in AOC3-GFP immunoprecipitates from plants transiently expressing both MPK4-HA and AOC3-GFP, but not in control plants only expressing MPK4-HA (Fig. 3). Thus, MPK4 and AOC3 can be found in complex in planta.

We also generated double transgenic Arabidopsis lines in the mpk4-1 background expressing MPK4 with a C-terminal HA tag under control of its native promoter and AOC3 tagged C-terminally with either Myc or GFP under control of a 35S promoter. MPK4-HA were detected in AOC3-Myc and AOC3-GFP immunoprecipitates only from double transgenic lines but not from control lines expressing only MPK4-HA (Fig. 4).
MPK4-HA is detected in AOC3-GFP immunoprecipitates from N. benthamiana transiently expressing AOC3-GFP and MPK-HA. Immunoblots of input and anti-GFP immunoprecipitates are probed with anti-HA and anti-GFP antibodies.

![Figure 3: MPK4 is in complex with AOC3 in planta.](image)

Figure 3: MPK4 is in complex with AOC3 in planta.

MPK4 is activated by treatment with the bacterial flagellin-derived flg22 peptide (Rodriguez et al., 2010). We questioned whether activation of *Arabidopsis* immune responses including MPK activation would affect the interaction between AOC3 and MPK4. We therefore pre-treated doubly transgenic plants expressing AOC3-GFP and MPK4-HA for 10 minutes with 100nM flg22 before immunoprecipitation of AOC3-GFP. MPK4-HA was detected in immunoprecipitations from both flg22 and mock treated plants (Fig. 5). A less intense band was observed in the immunoprecipitation from flg22 treated plants, but the experiment has not been repeated and therefore it is not possible to conclude if the lower intensity arises from stochastic events or if the affinity of the interactions decreases upon flg22 perception.

![Figure 4: MPK4 is in complex with AOC3 in Arabidopsis.](image)

Figure 4: MPK4 is in complex with AOC3 in Arabidopsis.

Immunoprecipitates from four-week-old plants expressing MPK4-HA and AOC3-GFP or AOC3-Myc. (A) MPK4-HA is detected in AOC3-GFP immunoprecipitates. Immunoblots of input and anti-GFP immunoprecipitates are probed with anti-HA and anti-GFP antibodies. (B) MPK4-HA is detected in AOC3-Myc immunoprecipitates. Immunoblots of input and anti-Myc immunoprecipitates are probed with anti-HA and anti-Myc antibodies.
Over-expression of AOC3 leads to increased resistance

While *mpk4-1* plants expressing MPK4-HA under its native promoter are complemented and thus indistinguishable from wild type overexpression of AOC3-Myc or AOC3-GFP in these plants caused dwarfism (Fig. 6A). *Mpk4* mutants also exhibit dwarfism and we therefore asked if overexpression of tagged AOC3 also caused accumulation of PR1 protein. Plants overexpressing AOC3-Myc accumulated PR1 protein to a similar extent as the *mpk4-1* mutant (Fig. 6B). AOC3-GFP overexpressing plants also accumulated PR1 protein but to a lesser extent and *mpk4* complemented lines had no detectable accumulation of PR1 protein (Fig. 6B). Since *mpk4* plants also exhibit increased resistance towards virulent pathogens, we next assayed the susceptibility of AOC overexpressing plants. Both AOC3-GFP and AOC3-Myc overexpressing plant exhibited increased resistance against *pst* DC3000 (Fig. 6C). Thus, overexpression of AOC3 interestingly leads to accumulation of PR1 protein and induced resistance against *pst* DC3000.

AOC3 is an MPK4 substrate

Since MPK4 and AOC3 are found in complexes *in planta*, it is likely that AOC3 also represent an MPK4 substrate. Therefore, we carried out *in vitro* kinase assays using immunoprecipitated flg22-activated MPK4 or MPK3 or MPK6 to determine whether AOC3 is also an MPK4 specific substrate. These MPKs were then incubated with recombinant His6-AOC3 protein lacking the chloro plast transit peptide. Phosphorylated His6-AOC3 was detectable as a radioactive band around 20 kDa after incubation with flg22-activated MPK4 (Fig. 7), while MPK6 caused only low levels of AOC3 phosphorylation and MPK3 did not significantly alter AOC3 phosphorylation. Each MPK
Figure 6: Overexpression of AOC3 induce resistance.

(A) Plants photographed at 4 weeks of growth in short day conditions, genotypes as indicated. (B) PR1 protein accumulation is elevated in mpk4-1 complemented lines overexpressing AOC3-myc and AOC3-GFP. Four-week-old plants were used for protein extraction and Bradford determined protein concentrations were adjusted to equal levels before SDS page (Samples are run in duplicates). Immunoblots were probed with anti-PR1. (C) Tagged AOC3 lines are more resistant to colonization by *Pst* DC3000 (OD600=0.001). Bacteria were syringe-infiltrated and samples taken 4 days post-infiltration. Data are shown as log10-transformed colony-forming units/cm² leaf tissue. Standard error of the mean is indicated by errors bars (n = 7). Statistical significance between the mean values was determined by ANOVA followed by Tukey’s test. Multiplicity adjusted P values are shown for significantly different mean values.
Figure 6: AOC3 is phosphorylated in vitro by MPK4
MPK-3, -4 and -6 were immunoprecipitated from extracts of Col-0 seedlings treated with 200 nM flg22 for 10 min. For the negative control (-), extracts were incubated with agarose beads without antibodies. Immunoprecipitates were incubated with His6-AOC3 or MBP for 60 min at 30°C before SDS-PAGE. Autoradiogram (top panel), Coomassie-stained gel for loading control (bottom).

was also incubated with the generic MPK substrate myelin basic protein (MBP) to verify their activation (Fig. 7). Thus, AOC3 is an in vitro substrate of MPK4, if this is also true in vivo is the subject of further research.

AOC3 contains 1 Ser-Pro (SP) and 1 Thr-Pro (TP) motif which are commonly phosphorylated by MPKs (Pearson et al., 2001; Ubersax and Ferrell Jr, 2007). The SP motif is conserved in all 4 Arabidopsis AOC enzymes (Fig. 2) and is central for their enzyme activity (Hofmann et al., 2006; Schaller et al., 2008). In AOC2 the SP motif (Ser-96,Pro-97) and two Asn residues (Asn-90,Asn-118) are important for stabilizing the reactive oxygen in the epoxide group in catalyzing the cyclization of 13-HPOT into OPDA (Hofmann et al., 2006; Schaller et al., 2008). Online database searches (PhosPhAt and P3DB) showed that AOC2 from Arabidopsis, as well as AOC4 from Glycine max (Soybean) and Medicago truncatula (barrel clover), are phosphorylated at this specific SP motif (Durek et al., 2010; Yao et al., 2014). It is reasonable to believe that this phosphorylation might regulate the activity of AOC enzymes as addition of the negatively charged phosphate group to the SP motif could affect the stabilization of the epoxide group in the 13-HPOT substrate. To test if MPK4 can phosphorylate the SP site we incubated a mutant version of His6-AOC3 with Ser-100 mutated to alanine (S100A) with MPK4 immunoprecipitates and measured the incorporation of radioactive labeled AT\textsuperscript{32}P (Fig. 8). There was no clear difference in the phosphorylation of AOC3 and AOC3-S100A meaning that (i) MPK4 does not phosphorylate AOC3-Ser100 in vitro or (ii) the
phosphorylation of an additional residue(s) mask the reduction in phosphorylation of the AOC3-S100A mutant.

To investigate the MPK4 mediated phosphorylation of AOC3 we have cloned, expressed and purified the additional phospho site mutants AOC3-T233A and AOC-S100A/T233A, but we have not yet tested their specificity as MPK4 substrates.

Figure 6: AOC3A100A is phosphorylated by MPK4.
MPK4 was immunoprecipitated from extracts of Col-0 seedlings treated with 200 nM flg22 for 10 min. For the negative control (-), extracts were incubated with agarose beads without antibodies. Immunoprecipitates were incubated with His6-AOC3 or His6-AOC3S100A for 60 min at 30°C before SDS-PAGE. Autoradiogram (top panel), Coomassie-stained gel for loading control (bottom).

**MPK4 associates with chloroplasts.**

AOC3 is transcribed in the nucleus, translated in the cytosol and an N-terminal transit peptide guides the protein to the chloroplast where it functions in JA biosynthesis (Schaller et al., 2008). MPK4 has previously been shown to be present in the cytoplasm and nucleus, but no association to plastids has so far been shown (Andreasson et al., 2005; Petersen et al., 2000, 4). A large scale phosphoprotein analysis discovered that numerous chloroplast proteins are phosphorylated at putative MAP kinase phosphorylation motifs (Reiland et al., 2009). However, no MAP kinase has been shown to associate with chloroplasts. We therefore questioned where the interaction between MPK4 and AOC3 occurs and investigated whether MPK4 associates with chloroplasts.

To answer these questions, we isolated intact chloroplasts from transgenic plants expressing MPK4-HA and wild type Ler plants and probed for MPK4-HA by immunoblotting (Fig. 9). A clear, distinct band for MPK4-HA was observed in the chloroplast fraction from MPK4-HA expressing plants, but was absent in the control plants. We further probed for the presence of MPK6, another immunity regulating MAPK (Rasmussen et al., 2012). MPK6 was detected only in the total protein
fraction and not in the chloroplast fraction (Fig. 9). To exclude the possibility that the chloroplast fractions were contaminated with cytoplasmic protein we probed for the cytoplasmic protein DECAPPING 1 (DCP1) (Fig. 9) (Xu et al., 2006, 2). We detected DCP1 only in the total protein sample and not in the chloroplast fractions. This indicates that these fractions where not contaminated with cytoplasmic proteins. We also probed for the presence of the chloroplast protein COPPER/ZINC SUPEROXIDE DISMUTASE 2 (CDS2) to verify presence of chloroplastic proteins (Fig. 9) (Pilon et al., 2011). CSD2 was observed in both total protein samples and the chloroplast fractions. Whereas CSD2 is present in the chloroplast, CSD1 is a cytoplasmic protein (Pilon et al., 2011). The CSD2 antibody cross-reacts with CSD1 and a lower band is observed in the total protein samples but is absent in the chloroplast fractions. This band correlates to the size of CSD1 (15 kDa). These results confirm that the chloroplast fractions are free of cytoplasmic proteins.

Figure 9: MPK4 is detected in isolated chloroplasts.
Intact chloroplasts were isolated from leaf tissue of four-week-old plants expressing MPK4-HA under control of its native promoter or from Ler wild type plants. Total protein samples and chloroplast fractions were then subjected to SDS-PAGE and immunoblotting.
MPK4 does not have a chloroplast transit peptide. However, proteomic data suggest that up to 30% of the protein species in the chloroplast lack encoded transit peptides and therefore are translocated to the chloroplasts by unknown mechanisms (Armbruster et al., 2009). It is therefore possible that MPK4 can translocate to the chloroplasts even in absence of a transit peptide. Alternatively MPK4 could also simply associate with the outer envelope of the chloroplast without actually being translocated into the chloroplast.

Discussion

In this study, we identify AOC3 as an interactor and substrate of the immunity regulating MPK4 in Arabidopsis. AOC3 is located in the chloroplasts where it catalyzes the production of the JA precursor ODPA (Hamberg and Fahlstadius, 1990). Although the enzymatic steps in the biosynthesis of JA are well understood, information regarding regulation is scarce. A possible point of regulation might be controlling the availability of enzymes necessary in the pathway. LOX2 catalyzes the first step in the JA biosynthesis by converting α-linolenic acid into 13(S)-HpOTrE (Stenzel et al., 2003). LOX2 interacts with the eukaryotic translational initiating factor 4E (eIF4E) in the cytosol (Freire et al., 2000). It is possible that this interaction sequesters LOX2 in the cytosol thwarting the initial step in JA biosynthesis. Alternatively, it is also possible that LOX2 actually down-regulates translation through its interaction with eIF4E. Confirming or rejecting one or both of these hypotheses requires further investigation.

Regulation of the pathway is also likely to occur through phosphorylation. Numerous sites of chloroplast proteins are mapped through phospho-proteome profiling including several motifs thought to be phosphorylated by MAP kinases (Reiland et al., 2009). Online database searches (PhosPhAt and P3DB) show that AOC2 from Arabidopsis as well as AOC4 from Glycine max (Soybean) and Medicago truncatula (barrel clover) are phosphorylated at a conserved SP motif (AOC3 Ser-100 Pro-101) (Durek et al., 2010; Yao et al., 2014). This SP motif is important for the function of the AOC enzymes as it functions in stabilizing the reactive oxygen of the epoxide group in its substrate when 13-HPOT cyclizes into OPDA (Hofmann et al., 2006; Schaller et al., 2008). This however does not seem to occur though the activity of MPK4 since an SP to AP mutant was still phosphorylated by MPK4 in vitro (Fig. 8). The database searches further showed that AOC4 from Medicago truncatula is phosphorylated on an SP site between α-helix 3 and α-helix 4. In Arabidopsis AOC1-3 has a TP site between α-helix 3 and α-helix 4 while AOC4 has an EP site putatively mimicking a phosphorylated motif (Fig. 2). It is likely that this motif can be phosphorylated by MPK4, although this and its possible effects have to be tested experimentally.
To gain insight into where the interaction between AOC3 and MPK4 occurs, we investigated if MPK4 associates with chloroplasts. MPK4 was clearly observed in immunoblots from isolated chloroplast (Fig. 9). This, however, does not provide information of whether MPK4 is translocated into the chloroplast or if it merely resides attached to the outer membrane. Therefore, to further explore the location of the molecular interaction between AOC3 and MPK4 studies such as bimolecular fluorescence could be applied for a more precise confirmation of where in the cell this interaction occurs. Nonetheless, the presence of phosphorylated MAP kinase motifs in chloroplast proteins sustains the idea of MPK4 functioning inside the chloroplast as well as in the cytosol and nucleus (Andreasson et al., 2005; Reiland et al., 2009). If MPK4 resides inside the chloroplast, it raises the question whether it is activated by perception of pathogens similar to its cytoplasmic counterpart? And, if this is the case, can MPK4 regulate JA biosynthesis through phosphorylation of AOC3 (and AOC1 and -2) upon pathogen perception?

MPK4 was initially considered to regulate JA induced gene transcription as the mpk4 mutant fails to accumulate JA induced PDF1.2 transcripts (Petersen et al., 2000, 4). However, crossing the mpk4 mutant into an eds1 mutant background which blocks ETI restored the exogenous JA induced accumulation of PDF1.2 transcripts (Brodersen et al., 2006). This implies that the blockage of JA induced genes in the mpk4 mutant is a pleiotropic effect of activating ETI and not necessarily linked to MPK4 lack of function. Further investigation of the phosphorylation of AOC3 by MPK4 may shed light on a putative regulatory point in JA biosynthesis and how this converges with defense signaling.

**Materials and methods**

See material and methods for “eIF4E-Thr6 is specifically phosphorylated in vitro by MPK4 and form a complex in the nucleus in vivo.”
References


eIF4E-Thr6 is specifically phosphorylated *in vitro* by MPK4 and form a complex in the nucleus *in vivo*.

**Introduction**

Translation of mRNAs into proteins is primarily regulated at the point of initiation (Jackson et al., 2010). Canonical translational initiation in most eukaryotes involves scanning of the 5’ untranslated region (UTR) for the start codon by a ribosomal preinitiation complex, which is comprised of the 40S ribosomal subunit end several eukaryotic initiation factors (eIFs) (Jackson et al., 2010; Topisirovic et al., 2011). A preliminary step in the formation of the preinitiation complex is the binding of eIF4F complex to the 5’cap of the mRNA (Merrick, 2015). The eIF4F complex comprise the proteins eIF4E which is the actual 5’cap binding protein, the RNA helicase eIF4A and the scaffold protein eIF4G (Merrick, 2015). At the center of this complex is eIF4G a multidomain protein, which functions as a scaffold protein for both eIF4E and eIF4A (Merrick, 2015). When eIF4G binds eIF4E it induce allosteric changes and enhance the affinity of eIF4E for the 5’cap of the mRNA (Gross et al., 2003; Volpon et al., 2006). Intriguingly the binding of the 5’cap of the mRNA also enhances the interaction between eIF4E and eIF4G, thus stabilizing the complex (Volpon et al., 2006). eIF4G also enhance the activity of the RNA helicase eIF4A and functions as a scaffold protein that induce allosteric conformational changes to both eIF4A and eIF4E (Gross et al., 2003; Schütz et al., 2008; Volpon et al., 2006).

eIF4G also interacts with eIF3 and poly-A binding protein (PABP). After the eIF4F complex has bound the 5’cap of the mRNA eIF3 recruits the 40S ribosomal subunit and several other eIFs resulting in the formation of the 48S ribosomal initiation complex that scan the 5’UTR for a transitional start site (Jackson et al., 2010; Topisirovic et al., 2011). The binding of PABP by eIF4G is thought to circularize the mRNA which is believed to enhance translation due to recycling of the ribosome after ended translation (Topisirovic et al., 2011).

Initiation of translation is somehow conserved in all eukaryotes although differences occur. For example, in contrast to the three-subunit eIF4F complex identified in mammals and plants, only a two-subunit complex comprising eIF4G and eIF4E have been identified in yeast. However, eIF4A helicase activity in yeast is believed to function in association with eIF4E and eIF4G (Mayberry et al., 2011; Merrick, 2015). Plants also stand out as in both yeast and mammalian cell lines, but no plants have eIF4E binding proteins (4E-BP) that regulate the availability of eIF4E by sequestration thereby inhibiting the formation of the eIF4F complex (Lukhele et al., 2013; Peter et al., 2015).
mammals and yeast phosphorylation of 4E-BPs functions to regulate translational initiation and phosphorylated 4E-BPs lose the affinity for eIF4E, promoting the formation of the eIF4F complex and thereby translation (Merrick, 2015). How plants regulate translational initiation in absence of 4E-BPs is unclear. In mammals, a MAPK-interacting kinase (MNK) phosphorylates eIF4E at a conserved residue (Ser-209) in the C-terminal region (Fig. 1). This phosphorylation upregulates translation of a subset of genes involved in inflammation responses and tumor progression (Furic et al., 2010). Using phosphoproteomic several other phosphorylated residues in human eIF4E has been identified, but no function has been assigned to these phosphorylated residues (Fig. 1) (PhosphoSitePlus Database: Eto, 2010; Franz-Wachtel et al., 2012; Hornbeck et al., 2015; Li et al., 2009; Rychlik et al., 1987; Sharma et al., 2014; Van Hoof et al., 2009; Zhou et al., 2013). The Ser-209 of human eIF4E does not align to any Ser or Thr residues in the Arabidopsis eIF4E (Fig. 1), so it is plausible that this specific phosphorylation is explicit for humans/mamals. Nonetheless one phosphosite has been identified in Arabidopsis eIF4E (Fig. 1), but no function has been assigned to it (PhosPhAt Database: Durek et al., 2010).

<table>
<thead>
<tr>
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<th>1 MAVE-----DTKSVTETAPPSIENPIDRYHEEDEEGDAECGGCPEDGNYDESSKSG</th>
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<tr>
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<td>1 MATVEPETPVPNPETEEBEPSKNO----------------------------------</td>
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<tr>
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<tr>
<td>Hs_eIF4E</td>
<td>210 ITRQRVV</td>
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Figure 1: Alignment of human and Arabidopsis peptide sequence
Sequences were aligned with T-Coffee and colored by BoxShade3.2. Known phospho-sites (PhosPhAt and PhosphoSitePlus) are indicated by red background coloring and putative MPK phospo-sites are marked above the alignment with (*).
The Arabidopsis genome contain four different eIF4E genes namely eIF4E (AT4G18040), eIF4E-B (AT1G29550), eIF4E-C (AT1G29590) and eIF(ISO)4E (AT5G35620). eIF4EB and eIF4EC are expressed at very low levels and are therefore not thought to function in general translation, but expression of eIF4EB is upregulated in flowering tissue implying a possible role in reproduction (Patrick et al., 2014; Rodriguez et al., 1998). eIF4E are expressed throughout all green tissue whereas eIF(iso)4E are expressed mainly in flowers (Rodriguez et al., 1998). Albeit differential expression patterns of eIF4E and eIF(iso)4E they are able to complement each other’s function in Arabidopsis as individual loss-of-function mutants reassemble a wild type phenotype whereas the double eif4e eif(iso)4e mutants are embryo lethal (Callot and Gallois, 2014). In Arabidopsis eIF4E and eIF(iso)4E interacts with eIF4G and eIF(iso)4G respectively forming eIF4F and eIF(iso)4F complexes (Patrick et al., 2012). It is therefore more likely that the complementation occurs through the eIF4F and eIF(iso)4F complexes. In vitro assays shows that eIF4(iso)4E has an up to 10 fold lower affinity for a 5’cap analog than eIF4E (Kropiwnicka et al., 2015). This raises the question of how these proteins compete with each other in vivo for in translational initiation or perhaps it is more appropriate to question whether they actually compete? Albeit they are able to complement each other meaning that they are both able to initiate general translation, it is possible that they both have favored affinity for a subset of differential mRNA species.

Some viruses depend on the host translational machinery for translation of their genome, and a few of these have been shown to rely specifically on either eIF4E or eIF(iso)4E. Thus, mutations in these proteins are often associated with loss of susceptibility (Sanfaçon, 2015). Genomic potyvirus RNA contains a 5′ end covalently linked virus-encoded protein (VPg), which is required for virus infectivity (Riechmann et al., 1992). The VPg linked to the viral genome interacts with the host translational machinery on which it is dependent on translation of the viral genome (Wang and Krishnaswamy, 2012). Therefore, mutations or loss-of-function of indispensable host factors required by the virus can thwart viral infection (Diaz-Pendon et al., 2004). So far all mutations in genes conferring recessive resistance in plants are found in eIFs and the vast majority are found in genes coding for eIF4E or eIF(iso)4E (Wang and Krishnaswamy, 2012). Pytoviruses display selective involvement of either eIF4E or eIF(iso)4E during infection for example Clover yellow vein virus (CLYVV) dependents on the interaction between the viral VPg and the host eIF4E and not eIF(iso)4E for proliferation and (Sato et al., 2005). Whereas both Turnip mosaic virus (TuMV) and Lettuce mosaic virus (LMV) on the other hand rely on interactions between their viral VPg and host eIF(iso)4E and not eIF4E (Duprat et al., 2002). In some cases the VPg compete with host 5’capped mRNAs for their interaction with eIF4E or eI4(iso)4E, but because the specific motifs required for
interaction differs for each pytoivirus. Thus in some cases eIF4E or eIF(iso)4E interacts with both host 5’capped mRNAs and viral VPg proteins. And therefore the is no consensus regarding whether or not domains for binding 5’capped host mRNA and viral VPg proteins overlap (Charron et al., 2008; Truniger and Aranda, 2009).

Immune signaling is often involve Mitogen Activated Protein kinases (MPKs). In Arabidopsis MPK3, MPK4 and MPK6 are described as canonical defense regulating kinases (Rasmussen et al., 2012). All three kinases are activated by perception of Pathogen Associated Molecular Patterns (PAMPs) by plasma membrane spanning Pathogen Recognition Receptors (PPRs). Activated MPKs can then transduce immune signaling through for example phosphorylation of defense related transcription factors (Rasmussen et al., 2012). In plants, virulent pathogens can inject effector protein directly into the host cell. These effectors can then modify the host to suppress immune responses. Both MPK3, MPK4 and MPK6 are pathogenic effector targets, implying that they are important immune regulators (Deslandes and Rivas, 2012). Successful plants have evolved resistance (R) proteins that can directly sense pathogenic effector proteins or indirectly recognize their effect. SUPPRESSOR OF MKK1 MKK2 1 (SUMM2) surveillances the MPK4 pathway and is activated in absence of MPK4 activity (Zhang et al., 2012). Activation of SUMM2 activates effector-triggered immunity (ETI) which is somehow similar to PAMP-triggered immunity (PTI), but defense response are both amplified and prolonged and can include localized programmed cell death to effectively thwart pathogen growth (Cui et al., 2015; Zhang et al., 2012).

A screen for MPK substrates using functional protein microarrays published by Popescu et al. (2009) identified 152 possible MPK4 substrates. However, by focusing on substrates phosphorylated only by MPK4 we could reduce this list to 14 potential MPK4 specific substrates. We scrutinized these 14 proteins and further eliminated three proteins that lacked the canonical Ser/The-Pro MPK phosphorylation site (Pearson et al., 2001). We cloned the remaining list of eleven proteins and co-expressed them in N. benthamiana with MPK4 to identify any possible interactions by co-immunoprecipitation. We identified eIF4E as an MPK4 interacting protein and further investigated its relation to MPK4 both in vitro and in Arabidopsis.
Results

eIF4E is an MPK4 substrate

In the MPK substrates published by Popescu et al. (2009) we focused on eIF4E as a putative substrate of MPK4 kinase activity. Not all substrates reported by Popescu et al. (2009) contained the canonical Ser/Thr-Pro MPK phosphorylation motif (Pearson et al., 2001). We therefore first performed an in vitro kinase experiment to confirm eIF4E as an MPK4 substrate. We immunoprecipitated PAMP activated MPKs and incubated them with recombinant His6-eIF4E protein purified from e.coli. Phosphorylated His6-eIF4E was observed as a radioactive band after incubation with flg22 activated MPK4, while nor MPK3 or MPK6 resulted in His6-eIF4E phosphorylation (Fig. 2). Each MPK was also incubated with the generic MPK substrate myelin basic protein (MBP) to verify their activation (Fig. 2). The eIF4E peptide sequence contains one putative MPK phosphorylation site (Thr6-Pro7). A version of eIF4E with Thr6 mutated to an alanine (eIF4E-T6A) was also incubated with immunoprecipitated MPK-3, -4 and -6, this mutant had significantly lower levels of phosphorylation after incubation with MPK4 (Fig. 2). Thus, eIF4E-Thr6 is phosphorylated in vitro by MPK4 and not by MPK3 or MPK6. This phosphorylated TP site aligns with a TP site in the human eIF4E, which is also phosphorylated (Fig. 1). The effect of phosphorylating this TP site in human eIF4E is unknown, but given its homology to the TP site in Arabidopsis eIF4E it might have a conserved regulatory function.

Figure 2: MPK4 specifically phosphorylates eIF4E in vitro.
MPK-3, -4 and -6 were immunoprecipitated from extracts of Col-0 seedlings treated with 200 nM flg22 for 10 min. For the negative control (-), extracts were incubated with agarose beads without antibodies. Immunoprecipitates were incubated with His6-eIF4E, His6-eIF4E-T6A or MBP for 60 min at 30°C before SDS-PAGE. Autoradiogram (top panel), Coomassie-stained gel for loading control (bottom).
eIF4E interacts with MPK4 in planta

To examine the interaction we generated transgenic Arabidopsis plants in an eif4e T-DNA loss-of-function line (SALK_145583) expressing eIF4E-Myc under control of a 35S promoter. In anti-Myc immunoprecipitations from transgenic eIF4E-Myc tissue, we detected a 40-kDa band corresponding to MPK4 (Fig. 3). We questioned whether a non-phophorylatable form of eIF4E would affect the interaction. We therefore generated transgenic plants expressing eIF4E-T6A-Myc under control of a 35S promoter in the eif4e T-DNA loss-of-function mutant. Anti-Myc immunoprecipitation from transgenic eIF4E-T6A-Myc tissue detected a more intense band than from wild type eIF4E-Myc (Fig. 3). Thus, the interaction between MPK4 and the non-phosphorylatable eIF4E-T6A is stronger than the interaction with eIF4E. This may indicate the interaction with MPK4 is dependent on whether or not eIF4E is phosphorylated.

![Figure 3: MPK4 is in complex with eIF4E eIF4E-T6A in Arabidopsis.](image)

Immunoprecipitates from five-week-old plants expressing eIF4E-Myc or the phospho-dead mutant eIF4E-T6A-Myc. MPK4 is detected in anti-Myc immunoprecipitates. Immunoblots of input and anti-Myc immunoprecipitates are probed with anti-MPK4 and anti-Myc antibodies.

**eif4e mutants does not exhibit autoimmunity similar to mpk4**

eif4e plants exhibit normal growth and are indistinguishable from Col-0 plants (Fig. 4A). Nevertheless, given that MPK4 is an important regulator of defense, we tested whether eIF4E may also be involved in immunity. To this end, we first examined for altered bacterial resistance by syringe infiltrating pst DC3000, into eif4e leaf tissue. However, there was no noticeable difference in bacterial growth after four days when compared to Col-0 (Fig. 4B). Given that eIF4E and eIF(iso)4E can complement each other, we hypothesized that if phosphorylation of eIF4E by MPK4 is important for defense then the non-phosphorylatable eIF4E-T6A might dominantly inhibit defense.
regulatory functions normally mediated by eIF4E. We therefore also tested for altered resistance in the eIF4E-T6A complementing lines by syringe infiltrating *pst* DC3000, but no noticeable difference was observed in bacterial growth after four-days post infiltration between these plants, Col-0 and eIF4E-Myc complementing lines (Fig. 4B)

This implies that phosphorylation of eIF4E-Thr6 is not critical for normal defense responses against *pst* DC3000, nonetheless it is possible that eIF4E might be involved in defense responses against other types of pathogens.

Figure 4: *eif4e* does not induce autoimmunity

(A) Photographs of five-week old plants, genotypes are as indicated.

(B) Four-week old plants we infiltrated with *pst* DC3000 (OD600=0.001). Bacteria were syringe-infiltrated and samples taken 4 days post-infiltration. Data are shown as log10-transformed colony-forming units/cm² leaf tissue. Standard error of the mean is indicated by errors bars (n = 4).
**ClYVV susceptibility is not dependent on eIF4E phosphorylation**

Potyviruses including ClYVV and TuMV seize the host translational machinery to proliferate themselves. ClYVV and TuMV depend on interactions with eIF4E and eIF(iso)4E respectively to initiate translation of their genomic RNA (Duprat et al., 2002; Sato et al., 2005). Thus, mutations in these genes can result in recessive resistance against viruses. We questioned whether phosphorylation of eIF4E by MPK4 would affect eIF4E activity and thus have impact on virus resistance against ClYVV. Given that *mpk4* exhibits autoimmunity and suppression by *summ2* is only partial, we tested for ClYVV susceptibility in *mkk1/2-summ2* plants. MKK1/2 are the upstream MPKKs of MPK4. The *mkk1/2* double mutant reassembles the phenotype of *mpk4*, however this phenotype is fully suppressed by *summ2*, while MPK4 is not activated by PAMP perception. Col-0 was susceptible to both ClYVV and TuMV, whereas respectively *eif4e* and *eif(iso)4e* were resistant (Fig. 5A+B).

![DAS ELISA ClYVV and TuMV](images)

**Figure 5**: ClYVV and TuMV susceptibility is not dependent on MPK4 activation. Five-week-old plants were inoculated with (A) ClYVV or (B) TuMV. Virus accumulation was assayed 24 dpi by ELISA. Error bars indicate SEM (n=6). The red line indicates threshold for susceptibility equal to three times the mean value for uninfected controls (n=3)
Both summ2 and mkk1/2-summ2 were susceptible to CIYVV and TuMV, thus lack of MPK4 activity do not impair eIF4E and eIF(iso)4E mediated CIYVV or TuMV proliferation.

It is possible that other kinases can phosphorylate eIF4E-Thr6 \textit{in vivo}, we therefore tested CIYVV susceptibility in the complementing eIF4E-T6A-Myc line. Plants expressing either eIF4E-Myc or eIF4E-T6A-Myc similar to \textit{Col-0} were susceptible to CIYVV (Fig. 6). We therefore conclude that absence of MPK4 mediated eIF4E phosphorylation does not affect eIF4E mediated CIYVV proliferation. Nevertheless, phosphorylation of eIF4E by MPK4 may regulate its activity in assays not tested here and might still be important for proper regulation.

\textbf{Figure 6: CIYVV susceptibility is not dependent phosphorylation eIF4E Thr6.}

Five-week-old plants were inoculated with (A) CIYVV. Virus accumulation was assayed after 24 days by ELISA. Error bars indicate SEM (n=6). The red line indicates threshold for susceptibility equal to three times the mean value for uninfected controls (n=3)
MPK4 and eIF4E interact in the nucleus.

Apart from its function in translational initiation eIF4E also function in export of mRNAs out of the nucleus, in both yeast and humans up to 68% of eIF4E is present in the nucleus (Iborra et al., 2001; Strudwick and Borden, 2002). eIF4E interacts with a secondary structure in the 3’UTR of specific mRNA and promotes their export out of the nucleus (Culjkovic et al., 2006). We were interested in where the MPK4-eIF4E interaction occurs in the cell and therefore employed a bimolecular fluorescence complementation assay (BiFC). We cloned MPK4 with a C-terminal fusion of nYFP and eIF4E with a C-terminal fusion of cYFP, both under control of a 3S promoter and co-expressed them transiently in Nicotiana benthamiana. When expressed together we detected distinct signal in the nucleus indicating reconstitution of YFP while no signal was observed when eIF4E-cYFP were co-expressed MPK6-nYFP (Fig. 7). When eIF4E-T6A-nYFP was co-expressed with MPK4-cYFP we observed an even more intense nuclear fluorescent signal compared to the eIF4E-nYFP (Fig. 7), which is in agreement with the CoIP data (Fig. 3). Thus, MPK4 and eIF4E interacts in the nucleus and the non-phophorylatable eIF4E-T6A gives rise to a stronger interaction.

Discussion

In this study, we characterize the translational initiation factor eIF4E as an interactor (Fig. 3+7) and substrate (Fig. 2) of the immune regulating kinase MPK4. MPK4 specifically phosphorylates eIF4E-Thr6, which align well with a phosphorylation site in human eIF4E (Fig. 1). Although no function has been assigned to this phosphorylation, it is possible that its function can be regulatory or serve as a (de)stabilizing factor. Given that MPK4 is an important immune regulator, we tested a possible role of eIF4E functioning in immunity. However we did not find evidence of eIF4E mediated resistance against pst DC3000 (Fig. 4B). Similar we did not find MPK4 activity (Fig. 5A) or phosphorylation of eIF4E-Thr6 (Fig. 6) important in conferring resistance against the potyvirys ClYVV that normally rely on eIF4E in Arabidopsis for its own proliferation. However, since we only tested a very limited set of pathogens we cannot exclude the possibility that phosphorylation of eIF4E can affect immunity in Arabidopsis.
Figure 7: eIF4E interacts with MPK4 in the nucleus

Confocal images of yellow fluorescent protein complementation. eIF4E-eYFP and eIF4E-T6A-eYFP were transiently co-expressed with either MPK4-nYFP or MPK6-nYFP in N. benthamiana. Confocal microscopy on leaf disks was conducted 2 days post-infiltration. Merged pictures show overlay of brightfield, chlorophyll and eYFP. Blue arrows indicate nuclei and the scale bar corresponds to 10 µm.
The interaction between MPK4 and eIF4E occurs in the nucleus (Fig. 7). Up to 68% of eIF4E is nuclei in both yeast and humans, in the nucleus, eIF4E serves different role than cytoplasmic translational initiation, instead eIF4E function in specific mRNA transport out of the nucleus (Culjkovic et al., 2006; Iborra et al., 2001; Strudwick and Borden, 2002). In humans overexpression of eIF4E leads to enhanced translation of a subset of mRNAs with long highly structured 5’UTRs. Interestingly these mRNAs are not upregulated at the transcriptional level, but rather actively transported out of the nucleus to the cytoplasm for translation (Culjkovic et al., 2007). In humans eIF4E mediated mRNA transport out of the nucleus depends on specific secondary structures in the 3’UTR distinct from bulk mRNA (Culjkovic et al., 2006). Thus, both structured 5’ and 3’UTR influence eIF4E mediated translation (Osborne and Borden, 2015). We show that in Arabidopsis, MPK4 interacts with eIF4E in the nucleus. It is therefore appealing to hypothesize that MKP4 can somehow regulate eIF4E mediated mRNA transport through phosphorylation. However, to this end we have no data showing eIF4E mediated transport in response to for example immunity. Further examination of the relationship between MPK4 and eIF4E are needed to clarify this hypothesis. Recently eIF4E has also been implicated in non-sense mediated decay (NMD) of mRNA in mammalian cells in response to insulin (Park et al., 2015). eIF4E seemingly interacts with the NMD factor Upstream Frameshift protein 1 (UPF1) as well as the general decapping factor Decapping 1 (DCP1) to promote NMD of eIF4E associated mRNAs (Park et al., 2015). We have recently shown that MPK4 interacts with and phosphorylates the mRNA decay factor Protein Associated with Topoisomerase II (PAT1) (Roux et al., 2015). PAT1 interacts with the cytoplasmic immune-receptor SUMM2 which in absence of PAT instigates ETI (Roux et al., 2015). Similar, ETI is also observed in NMD deficient mutants including upf1, suggesting a role of NMD in immunity (Jeong et al., 2011; Rayson et al., 2012). Any possible link between MPK4 mediated phosphorylation of eIF4E in Arabidopsis and mRNA decay are however still unclear and will be a subject for further investigation.

Materials and methods

Plants growth conditions

Arabidopsis thaliana Col-0 accession was used as wild-type in all experiments, except if otherwise stated. Salk lines were obtained from the Nottingham Arabidopsis Seed Centre (NASC). Lines obtained from NASC include SALK_101850 (aoc3), SALK_145583 (eif4e). mkks1/2 summ2 was a kind gift from Yuelin Zhang. Arabidopsis plants were grown in 9 × 9 cm pots at 22°C with a 8-h
photoperiod, or on plates containing Murashige–Skoog (MS) salts medium (Duchefa), 1% sucrose and 1% agar with a 16-h photoperiod.

**SDS-PAGE and immunoblotting**

SDS-tris/glycine or bis/tris gels were prepared with either 8, 10 or 12% acrylamide. Proteins were loaded and gels run at 100–150 V until the dye front reached the end of the gel. Gels were next electroblotted onto PVDF membranes (GE Healthcare) by wet transfer for 1h at 70 V. Membranes were rinsed in TBS and blocked for 30 min in 5% (w/v) non-fat milk in TBS-Tween (0.1% (v/v)). Membranes were incubated with primary antibodies for 1 h to overnight. Membranes were washed 3 × 10 min in TBS-Tween (0.1%) before 1-h incubation in secondary antibodies. Membranes were incubated in chemiluminescent substrate (100 mM Tris/HCl pH 8.8, 1.25 mM luminol, 5.3 mM hydrogen peroxide and 2 mM 4IPBA) (Haan and Behrmann, 2007) before exposure to a Sony a7S DSLR camera (Khoury et al., 2010). For AP-conjugated secondary antibodies membranes were incubated in NBT/BCIP (Roche) until bands were visible. For probing immunoblots with multiple antibodies, stripping was carried out using Restore Western Blot Stripping Buffer (Pierce) for 15–30 min, following three washes with TBS-Tween (0.1% (v/v)).

**Antibodies**

Primary antibodies were diluted in 1% (w/v) non-fat milk in TBS-Tween (0.1% (v/v)) to the following dilutions:

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Secondary antibodies were diluted in 1% (w/v) non-fat milk in TBS-Tween (0.1% (v/v)) to the following dilutions:

- anti-mouse-HRP 1:10,000 (Promega)
- anti-Rabbit-HRP 1:10,000 (Promega)
- ProteinA-HRP 1:1000 (GE Healthcare)

**Bacterial infection assays**

Pseudomonas syringae pv. tomato DC3000 (Pst DC3000) strains were grown in overnight culture in NYG medium supplemented with appropriate antibiotics. Cells were harvested by centrifugation and pellets resuspended in sterile water to indicated concentrations. Bacteria were infiltrated with a needleless 1-mm syringe into adult leafs and maintained in growth chambers for 3-4 days. Samples were taken using a cork-borer (6.5 mm) to cut leaf disks from infected leafs. Leaf disks were ground in water, serially diluted and plated on NYG plates with appropriate selection. Plates were incubated at 28°C and colonies counted 2–3 days later.

**Transient expression in Nicotiana benthamiana**

Agrobacterium tumefaciens strains were grown in LB medium supplemented with appropriate antibiotics overnight. Cultures were spun down and resuspended in 10 mM MgCl2 to OD600 = 0.8. For co-infiltrations bacterial cultures were mixed 1:1. Bacterial cultures were syringe-infiltrated into 3-week-old N. benthamiana leaves. Samples for protein extraction were harvested 2 days post-infiltration.

**Protein extraction and immunoprecipitation in Nicotiana benthamiana and Arabidopsis**

Plant material were ground in liquid nitrogen and extraction buffer [10 mM phosphate pH 7.2, 150 mM NaCl, 5 mM DTT, 0.1% Nonidet P-40, supplemented with EDTA-free protease inhibitor cocktail form Roche or 110 mM potassium acetate, 100 mM NaCl, 2 mM MgCl, 0.5%(v/v) TritonX-100 supplemented with EDTA-free protease inhibitor cocktail form Roche]

Samples were clarified by 10 min centrifugation at 4°C and 13,000 rpm. Supernatants were adjusted to 2 mg/ml protein and incubated 1-2 h at 4°C with GFPTrap-A beads (Chromotek), anti-Myc magnetic beads (Miltenyi Biotech) or EZview™ Red Protein A Affinity Gel supplemented with specific antibodies. Following incubation, beads were washed four times with extraction buffer, before adding 6XSDS loading buffer [300 mM Tris HCL pH 6.8, 300 mM DTT, 6% SDS, 6 mM EDTA, 0.03%(w/v) bromphenol blue, 60%(v/v) glycerol] and heating at 70°C for 10 min.
Purification of X7 polymerase
E.coli containing a plasmid encoding His tagged PfuX7 under control of a IPTG inducible promotor was grown in liquid LB media (Nørholm, 2010). At OD600=0.4 IPTG was added to a final concentration of 0.5mM. Cells where harvested by centrifugation and re-suspended in lysis buffer [] and lysed by 3-4 freeze/thaw cycles. Soluble protein were purified nickel spin columns () according to manufactures instruction. The enzyme was desalted by dialysis o.n. against storage buffer [50% glycerol, 100 mM Tris/HCl pH 8.0, 0.2 mM EDTA, 2 mM DTT, 0.2% NP40, 0.2% Tween 20.] and stored at -20C.

Genotyping
Leaf tissue was used for Edwards DNA extraction (Edwards et al., 1991). PfuX7 polymerase were used for genotyping PCR reactions in with a final concentration of 0.4 mM dATP, 0.4 mM dTTP, 0.4 mM dCTP, 0.4 mM dGTP, 20 mM Tris/HCl pH 8.8, 10 mM KCl, 6 mM (NH4)2SO4, 2 mM MgSO4, 0.1 mg/ml BSA and 0.1% Triton X-100 (Nørholm, 2010). Cycling conditions included a 60 second 96°C template denaturation step followed by 30 cycles of 15 second 56-65°C annealing step and a 15 second long primer extension step at 72°C. PCR products were electrophoresed on a 1% agarose gel in 0.5X TBE [45 mM Tris-borate/1 mM EDTA] with etBr and PCR products were visualized by UV light. Primers designed using the iSect tool (http://signal.salk.edu/isectprimers.html) were used to genotype lines for homozygosity.

Cloning and transgenic lines
Phusion Hot Start II High-Fidelity polymerase (Thermo) or PfuX7 (Nørholm, 2010) were used to amplify PCR products for all subsequent cloning reactions.

MPK6
MPK6 was cloned into pETNR D-TOPO (Invitrogen) from genomic DNA without stop codon with the following primers fwd “5’caccATGGACGCTGGTTCTCAGGTC” reverse “5’TTCGCTGATATTCTGGATGAAACG”

AOC3
AOC3 without signal peptide was cloned into pETNR pOPINf for expression in E.coli from cDNA DNA without start codon with the following primers fwd “5’AAGTTCTGGTTCAGGGCCACAGACACACGATTTATTACTTAACTCAA” reverse “5’ATGGTCTAGAAGCGGCTTTAATTAGTAAAGATACTTATAACTCCA”. For cloning AOC3-S100A the following start primer where used “5’AAGTTCTGGTTCAGGGCCACAGACACACGATTTATTACTTAACTCAA” reverse “5’AAGTTCTGGTTCAGGGCCACAGACACACGATTTATTACTTAACTCAA” reverse
AACGAAGGAGATAGAAACgcTCCAGC. For cloning AOC3-T235A the following reverse primer were used
“5’ATGGTCTAGAAAGCTTTAATTAGTAAAGTTACTTTATAACTCCACTAGGCTCCATCGCCTTAGCTTCCGGTGCCGTTTTCACATCCTTCGACGACGCAACCGCG”.

eIF4E

eIF4E was cloned into pETNR D-TOPO from genomic DNA without stop codon with the following primers fwd “5’caccATGGCGGTAGAAGACACTCC” reverse “5’AGCGGTGTAAGCCTTTTGGT “. For cloning eIF4E-T6A the following fwd primer was used “5’caccATGGCGGTAGAAGACgCTCCCAAATC”. eIF4E was cloned into pOPINf for expression in E.coli from cDNA without start codon with the following primers fwd “5’AAGTTCTGTTCAGGGCCCGCGGTAGAAGACACTCCAAA” reverse “5’ATGGTCTAGAAAGCTTTAAGCGGTGTAAGCGTTTTGTC”. For cloning eIF4E-T6A the following start primer was used “5’AAGTTCTGTTCAGGGCCCGCGGTAGAAGACgCTCCCAAATC”.

MPK6, AOC3 and eIF4E were recombined into binary gateway destination vectors for expression in plants using LR Clonase II (Invitrogen). For expression of C-terminally tagged GFP, HA, Myc, nYFP and cYFP construct pENTR clones were recombined into pGWB605, pGWB615, pGWB617, pcYCw and pnYGW respectively (HINO et al., 2011; Nakagawa et al., 2007). Clones were transformed into A. tumefaciens strain GV3101 and used for transient expression in N. benthamiana or floral dipping in Arabidopsis.

Recombinant protein purification and in vitro kinase assays

For in vitro experiments, PAT1 protein was purified from E. coli. The AOC3 and eIF4E CDS was cloned into pET15b (for an N-terminal His fusion) and transformed into E. coli BL21 (pLysS). Protein expression was induced by overnight treatment with 0.5 mM IPTG at 37°C, added to cells at OD600 = 0.6. Proteins were purified under native conditions Ni-NTA spin columns (Qiagen). For kinase assay see Roux et al. (2015).

Virus inoculation and detection by ELISA

TuMV and CIYVV were propagated on turnip (Brassica rapa) and Nicotiana benthamiana before virus inoculating of five-week-old Arabidopsis plants. Virus accumulation was assayed after 24 days by ELISA using respective viral antisera (DSMZ). Six plants per genotype were used in each
experiment. The susceptibility threshold is indicated for each experiment as a red line and refers to an absorbance value at 405 nm in ELISA equal to three times the mean value for uninfected controls.

References


Concluding remarks

MPK4 was initially characterized as a negative regulator of defense, due to MPK4 the loss-of-function mutant constitutively induction of defense responses (Petersen et al., 2000). MPK4 is activated by perception of the flagellin-derived flg22 peptide by the pathogen recognition receptor FLS2 (Rodriguez et al., 2010). FLS2 and its co-receptor BAK1 sequentially signals the activation of the three tiered MAPK signaling cascade comprising MEKK1, MKK1/2 and MPK4 (Chinchilla et al., 2007; Gao et al., 2008; Qiu et al., 2008a). Inactive MPK4 is bound to MKS1 and the WRKY33 transcription factor, upon activation, MPK4 phosphorylates MKS1, which release WRKY33 from the complex to induce transcription of the defense gene PAD3 (Andreasson et al., 2005; Qiu et al., 2008a, 2008b). The function of MPK4 as a negative regulator of defense is in contrast to MPK4 positively inducing transcription of PAD3. The MPK4 loss-of-function defense phenotype is suppressed my loss-of-function of EDS1 (Brodersen et al., 2006), which is essential for fully functional effector-triggered immunity (Falk et al., 1999). This indicated that the mpk4 defense phenotype was due to inappropriate activation of ETI and not due to loss of negative suppression of defense. Zhang et al. (2012) identified the R-protein SUMM2, which surveillance the MPK4 pathway and induce ETI upon disruption of this pathway. This endorse the hypothesis of the mpk4 defense phenotype arising from inappropriate activation of ETI and enables the analysis of the true in vivo function of MPK4 without pleiotropic effects in the mpk4 loss-of-function mutant.

In the previous sections, we have described and characterized three novel MPK4 substrates (i) PAT1 a protein functioning in decapping of mRNAs which was previously undescribed in plants. (ii) AOC3 functioning in JA biosynthesis and (iii) eIF4E with a dual function in both translational initiation and export of specific mRNAs out of the nucleus. Both PAT1 and AOC3 seems to be regulated through immune signaling whereas we were not able to position eIF4E in immunity. PAT1 is particular interesting given that its loss-of-function defense phenotype is dependent on SUMM2. PAT1 and SUMM2 interacts in planta and it is possible that SUMM2 guards a specific branch in MPK4 mediated immunity comprising PAT1. Equally interesting is the link between MPK4 and the JA biosynthesis component AOC3. The possible regulatory role that MPK4 have on AOC3 is still not clear, but the in vivo interaction between these two proteins prompted us to test if MPK4 is present in chloroplast. To this end, we have shown that MPK4, as the first MAPK so far shown, associates with the chloroplasts. For eIF4E, we have not found a putative role in MPK4 mediated defense, however we localized the interaction to take place in the nucleus. Given the dual function of eIF4E in both translation and mRNA export, it is still not clear what its connection to MPK4 is, but it is possible that MPK4 regulate either translation or mRNA export or both in
response to pathogens. With the findings presented here in this thesis, MPK4 is evidently a multifunctional MAPK functioning spatial and temporal in numerous cellular processes.

References


