Modulation of MAPK signaling in plant immunity and development

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Modulation of MAPK signaling in plant immunity and development

Modulering van MAPK signaaltransductieroutes betrokken
bij de immuunrespons en ontwikkeling van planten

(met een samenvatting in het Nederlands)

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Prof.dr. A.J.R. Heck

Voor mijn vader

Though leaves are many, the root is one;
Through all the lying days of my youth
I swayed my leaves and flowers in the sun;
Now I may wither into the truth
W.B.Yeats

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CHAPTER 1

General Introduction

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GENERAL INTRODUCTION

An underground balancing act between innate immunity and root growth

Most plants are sessile organisms and this lifestyle requires adaption to environmental conditions. They have developed sophisticated sensory mechanisms, allowing them to perceive and respond to exogenous (pathogen-derived) or endogenous (plant-derived) stimuli. Their defense system is able to distinguish between self and non-self and prevents potential infections by micro-organisms. This innate ability to perceive pathogenic microbes is a fundamental mechanism shared between plant and animal immunity. Innate immunity responses are evolutionary conserved and show striking similarities between plants and non-vertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Nurnberger et al., 2004; Ausubel, 2005; He et al., 2006)

In recent years exciting discoveries described the two strategies evolved by plants to combat pathogens. Microbes need to access the plant interior to become pathogenic, however after arrival at the host cell plasma membrane they encounter extracellular surface receptors or pattern-recognition receptors (PRRs), which recognize microbial elicitors or pathogen-associated molecular patterns or microbe-associated molecular patterns (PAMPs or MAMPs) (Chisholm et al., 2006; Schwessinger and Ronald, 2012). PRRs also perceive endogenous signals from the plant itself after a pathogenic attack, described as damage–associated molecular patterns (DAMPs) (Boller and Felix, 2009; Dodds and Rathjen, 2010). Basal or PAMP-triggered immunity (PTI) is suppressed by effector molecules released by virulent pathogens and in turn plants have evolved specific recognition machineries to perceive and detect such effector molecules (Chisholm et al., 2006). The direct or indirect activation of effector receptors induces effector-triggered immunity (ETI) (Chisholm et al., 2006; Schwessinger and Ronald, 2012).

Although immune responses may protect plants from pathogen attack, prolonged or de-regulated immune system activation has a negative impact on plant growth and development. Therefore plants need to allocate their resources and integrate growth and development with stress responses, including immune responses. Environmental cues target the biosynthesis or perception of growth hormones, which not only orchestrate intrinsic developmental programs, but also convey environmental input (Depuydt and Hardtke, 2011). For example, PAMP-triggered immune response was shown to inhibit the perception of the growth hormone auxin by down regulating the expression of proteins required to sense auxin levels (Navarro et al., 2006). Conversely, the attenuation of PTI has recently been connected to peptide hormone signaling (Igarashi et al., 2012; Mosher et al., 2012). Peptide hormones and their receptors are a relatively recent addition to the classic mobile growth regulators such as auxins, cytokinins and gibberellins (Matsuzaki et al., 2010). An on-going question in root development is how these phytohormones orchestrate and integrate environmental stimuli into developmental programs. Environmental signals influence the root architecture, therefore adaption of the intrinsic developmental programs is of pivotal importance for plant fitness.

Root meristem development and maintenance

Plant root system development is an important agronomic trait, as their root structure allows plants to provide anchorage in the soil and acquire the essential nutrients and water uptake. The root system consists of a primary root, which is extended throughout their lifecycle by branching. Plants have the unique property to grow and continuously develop during their lifelong cycle by elaborating their basic body plan determined in the embryo. In vascular plants there are five types of meristems: the shoot apical meristem (SAM), axillary meristem (AM), root meristem (RM), lateral/secondary meristem and the cambial meristem. Plant meristems generate the majority of the plant body by continuously initiating the formation of new organs and at later stages of development, flower meristems are formed that produce all floral organs (Stahl and Simon, 2012).

During the postembryonic growth phase the stem cells bring forth the cells of the primary meristems at opposite ends, at the apical side the SAM and at the basal end the RM (Stahl and Simon, 2009). In the RM all stem cells are internally organized around a small number of mitotically less active cells in the quiescent center (QC). The QC maintains stem cells in the *Arabidopsis thaliana* (Arabidopsis) root and defines the stem cell niche (SCN) (Aida et al., 2004). In higher vascular plants a mid-longitudinal section through the RM displays an arrangement of concentric columns or cell files each with a separate lineage affiliation. The main layers are from outside to inside designated as the lateral root cap, the epidermis, cortex, endodermis and pericycle cell files that surround the central vascular tissue in the middle of the root (Dolan et al., 1993; Benfey and Scheres, 2000).

In the first five days after germination the root grows with new cells arising through controlled stem cell divisions in the SCN and these cells migrate to a zone of cell division (meristematic zone). Next, the stem cell daughters cross through the zone of cell expansion without divisions, followed by the elongation-differentiation zone (EDZ), after which they differentiate to acquire tissue specific characteristics (Fig. 1). Cell differentiation is initiated in the transition zone (TZ), encompassing the boundaries between dividing and expanding cells in the different files (Fig.1, (Dello Ioio et al., 2007)). A reduction in the SCN activity may lead to a decrease in meristem size, caused by a loss of division potential of meristematic cells in the proximal meristem, or by a more rapid transit of the meristematic cells at the TZ to the EDZ (Dello Ioio et al., 2007). Thus, a balance of cell differentiation and the rate of generation of new cells may establish root meristem maintenance. Understanding the mechanism controlling this balance is a key question in plant development and in the next part, I will discuss briefly the phytohormones auxin and cytokinin, whose interactions in controlling meristem activity and therefore meristem maintenance, are important for understanding root development in Arabidopsis.

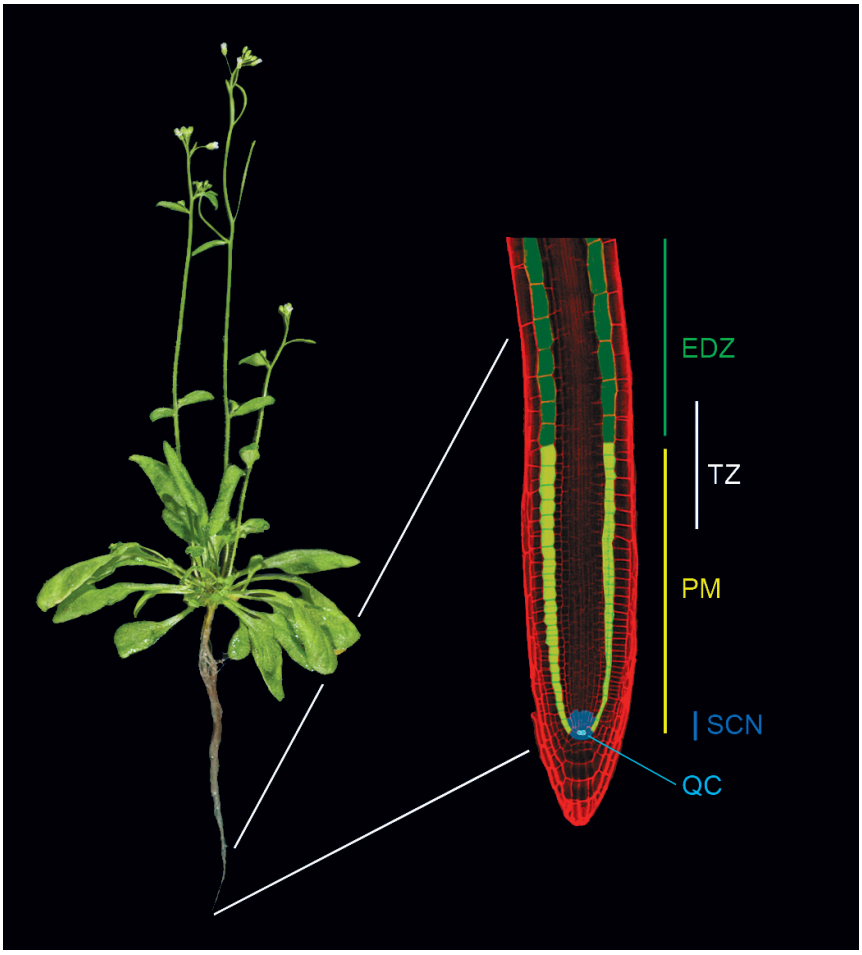

Figure 1. Schematic representation of the Arabidopsis primary root meristem. The stem cell niche (SCN) is marked in blue, the quiescent center (QC) or organizing center is marked in cyan blue. The cortex cell file of the proximal meristem (PM) is marked in green and the cortex cell file of the elongation-differentiation zone (EDZ) is marked in darker green. The transition zone (TZ) is the zone where cells prepare to leave the proximal meristem and enter the EDZ.

Phytohormone auxin modulates the primary root meristem

Phytohormones integrate environmental information into developmental pathways to determine plant shape (Teale et al., 2008). Eight principal classes of plant hormones have been characterized: abscisic acid, auxin, brassinosteroids, cytokinins, ethylene (ET), gibberellins, jasmonates (JA) and strigolactones. Among them, auxin mediates plant growth in response to environmental cues (Finet and Jaillais, 2012). Factors that regulate plant development have been studied for many years. As early as 1880, Charles Darwin described from an evolutionary perspective how growing plants adapt to different external stimuli,

including sensitivity to light (phototropism) and gravity (geotropism) of roots and shoots. It was not until 1937, that F.W. Went observed that tropic curvature requires the asymmetric distribution of the hormone auxin (Went, 1974) and identified auxin as the key regulator in plant growth and movement.

The cellular response to auxin is mediated by the F-box protein transport inhibitor response 1 (TIR1) and its homologues, which are subunits of the Skp1, Cullin and the F-box protein ligases (SCF) complex. The binding of auxin to the TIR1 auxin receptor and the F-box subunit of the $SCF^{TIR1}E3$ ubiquitin ligase promotes the ubiquitination and proteasome-mediated degradation of auxin/indole-3-acetic acid inducible genes (*AUX/IAA*) (Kepinski and Leyser, 2004; Dharmasiri et al., 2005). The targeted AUX/IAA degradation is the key event in auxin signaling, since it releases the repressive hold of the interacting auxin response factors (ARFs) and permits activation of transcriptional responses (Quint and Gray, 2006). The appropriate distribution of auxin is essential for normal plant development and particularly formation of an auxin gradient is necessary for cell specification within the root meristem (Sabatini et al., 1999; Friml et al., 2003; Grieneisen et al., 2007). Polar auxin transport (PAT) occurs in a cell-to-cell dependent manner, requiring specific influx and efflux carrier proteins that facilitate the uptake and release of auxin from /to the apoplast (Friml et al., 2003). The PIN-FORMED (PIN) proteins (Galweiler et al., 1998) are the most extensively studied proteins involved in PAT (Paponov et al., 2005). Single and multiple *pin* mutants show dramatic shoot and root meristem phenotypes (Leyser, 2005; Paponov et al., 2005), demonstrating that PAT is instrumental to meristem maintenance.

The importance of PAT in regulation of plant growth and development was also observed in the Arabidopsis bushy and dwarf 1 (*bud1*) mutant encoding *MAP KINASE KINASE7* (*MKK7*) (Dai et al., 2006). Expression of *MKK7* leads to a reduction in apical dominance and affected the plant architecture in *bud1,* displaying a dwarf phenotype with significant more branches. Disruption of auxin transport affects both the shoot as the root formation (Casimiro et al., 2001; Fukaki et al., 2002) and also a reduction in lateral root formation was observed in *bud1*.

The auxin/cytokinin interaction in the root meristem

Extensive genetic, phenotypic and molecular analyses in Arabidopsis revealed that cytokinins and auxin are key players in the control of cell division and differentiation in the primary root apical meristem (Hwang et al., 2012). Cytokinins are essential plant hormones that have a role in plant growth from early embryo development (Muller and Sheen, 2008). Various developmental functions have been described, including de novo organ formation from cultured tissues (Skoog and Miller, 1957) and release from apical dominance in shoots and roots ((Hwang et al., 2012) and references therein). Through interaction with other hormones, cytokinins regulate cell proliferation, while gibberellins promote cell elongation and auxin is involved in both processes (Depuydt and Hardtke, 2011).

The complex interaction between auxin and cytokinin controls cell division and differentiation in the primary root apical meristem (Perilli et al., 2010; Bishopp et al., 2011). This interaction balances cell differentiation with cell division, by controlling in opposite ways the abundance of the SHORT HYPOCOTYL 2 (SHY2) protein, a negative regulator of auxin signaling. Cytokinin reduces auxin response by activating Arabidopsis His kinases (AHK3)/ Arabidopsis response regulators (ARR1), a two-component signaling pathway resulting in transcription of the *IAA3*/*SHY2* gene. The SHY2 protein in turn negatively regulates the expression of the PIN genes and proteins (Dello Ioio et al., 2008). Conversely, auxin controls root meristem growth by directing degradation of the SHY2 protein, thus sustaining the activity of the *PIN* genes and cell division (Dello Ioio et al., 2008). Taken together, the establishment of root meristem size is determined by the antagonistically interaction at the vascular tissue transition zone by cytokinin and auxin and controls root growth.

Peptide hormone modulation of root meristem maintenance

In addition to classical phytohormone regulation of RM maintenance, recent evidence has emerged suggesting the involvement of peptide hormone signaling as well. The observation that the plant root meristem consists of a highly ordered cell arrangement generated by specific stem cells, combined with a quiescent center that regulates stem cell behavior, lead to the search for peptide molecules mediating intercellular communication to coordinate cell division and differentiation patterns (Stahl and Simon, 2012). In animal systems intercellular communication is important for the development and function of organs. Peptides are considered the most common mediators of cell-to-cell interaction in animal systems and this could be due to the diversity in length and their sequence, which are further diversified by posttranslational modifications (Matsubayashi and Sakagami, 2006).

Phytosulfokines were among the first peptide hormones identified in plants and are derived through proteolysis of larger precursors. PHYTOSULFOKINE (PSK) is a disulfated 5-amino acid secretory peptide and was identified as a mitogenic factor from asparagus mesophyll cells (Matsubayashi and Sakagami, 1996). While PLANT PEPTIDE CONTAINING SULFATED TYROSINE 1 (PSY1) is a mono-sulfated 18-amino acid glycopeptide, that was also identified in plant cell cultures (Amano et al., 2007). Both peptides PSK and PSY1 modulate cellular proliferation and differentiation and depend on tyrosine sulfation for their biological activity. Tyrosine sulfation, was first described in 1954 by Bettelheim et al., in bovine fibrinopeptide B and is a posttranslational modification restricted to proteins that transit the secretory pathway ((Moore, 2009) and references therein). The enzyme activity responsible for this modification is tyrosyl protein sulfotransferase (TPST), which catalyzes the transfer of sulfate from 3'-phosphoadenosine 5'-phosphosulfate to the phenolic group of tyrosine (Komori et al., 2009). Arabidopsis TPST (AtTPST) is a 62-KDa Golgi transmembrane localized protein (Komori et al., 2009).

The TPST protein is expressed throughout the plant body, however the highest levels of expression are found in the root apical meristem. Mutant *tpst-1* displays a pleiotropic

phenotype accompanied by a severe short- root phenotype and smaller cotyledons (Komori et al., 2009). Because *TPST* is a single-copy gene, a loss-of-function mutant likely reflects the deficiency in the biosynthesis of all the functional tyrosine-sulfated peptides found in Arabidopsis (Matsuzaki et al., 2010). Interestingly exogenous application of the peptides PSK and PSY1 to *tpst-1* seedlings was capable of restoring cell-elongation activity in the EDZ of the root (Matsuzaki et al., 2010)**.** The recognition of PSK by a membrane-localized leucine-rich repeat (LRR) receptor kinase, PSKR1 leads to the promotion of cellular proliferation (Matsubayashi et al., 2006). However disruption of PSKR1 and its two homologues PSKR2 and PSY1R in Arabidopsis leads to severe growth defects such as, stunted root growth, smaller leaves and early senescence (Amano et al., 2007). In other words, these phytosulfokines have a possible role in the root meristem in regulating the cellular capability to proliferate and differentiate.

A related class of tyrosine-sulfated peptides, the root meristem growth factors (RGFs), was recently identified as plant peptides involved in maintenance of the postembryonic root stem cell niche and meristem activity. They are mainly expressed in the stem cell area and in the first layer of columella cells (Matsuzaki et al., 2010; Perilli et al., 2011). While PSK and PSY1 are capable of restoring cell-elongation activity in the RM of *tpst-1*, RM architecture and RM length were partially restored in *tpst-1* mutant roots grown in the presence of RGF1 peptide. Furthermore, addition of PSK together with RGF1 fully restored root meristem length and root growth (Matsuzaki et al., 2010). This shows that both classes of tyrosine sulfated peptides are required for meristem maintenance and root growth.

A subset of RGFs was independently identified as GOLVEN (GLV) and is encoded by a family of genes, which alter root gravitropism when overexpressed (Whitford et al., 2012). Expression analysis of *GLV* lines using a homozygous single-locus *GLV* promoter:GUS transcriptional fusion showed, that not all *RGFs* function in the root (Whitford et al., 2012). Overexpression of GLV peptides in the primary root causes the formation of irregular waves (‘‘golven’’), including loops suggesting these peptides cause agravitropism. Whitford et al. (2012) described that GLV/RGFs are involved in the modulation of auxin-directed root bending and patterning through regulation of PIN2–mediated PAT distribution. In other words, GLV peptides are involved in controlling trophic growth.

Although these secretory peptides are required for various aspects of plant growth and development, currently only partial information of the processes regulating peptide-ligand receptor pathways is available. Recently the peptide ligand–receptor system of INFLORESCENCE DEFICIENT IN ABSCISSION (IDA), a secreted protein that acts as a putative ligand for a receptor-like protein kinase HAESA (HAE) and HAESA-LIKE2 (HSL2), was connected with a MAPK signaling module. This signal pathway from the putative ligand to the receptors and the cytoplasmic effector proteins regulates the abscission of floral organs (Cho et al., 2008) and is therefore important for development in Arabidopsis. Unraveling peptide-based signaling pathways however remains technically challenging, due to genetic

redundancy and future studies will elucidate the downstream effector proteins of the GLV/RGF pathway.

Unexpectedly, apart from their role in root meristem development, tyrosine-sulfated peptides have recently been linked by two studies to repression of immunity (Igarashi et al., 2012; Mosher et al., 2012). Mutations in either PSKR or TPST enhanced PTI against bacterial pathogen *Pseudomonas syringae*, while treatment with growth promoting peptide hormone PSK attenuated PTI signaling. These observations suggest that tyrosine-sulfated peptide signaling is important for maintaining optimal fitness through the correct balance between growth and immune response.

An introduction to innate immunity in plants

To cope with the risk of infection, plants and animals rely on different types of immune responsive systems: the "adaptive" immunity system found only in vertebrates and the more ancient "innate" immunity system, which is conserved in plants and in animals. In plants, two forms of innate immunity can be distinguished, basal or horizontal resistance and *resistance* (*R*) gene-based or vertical disease resistance (Boller and Felix, 2009). Basal resistance is initiated upon perception of potential microbial pathogens through recognition of PAMPs by plasma membrane localized PRRs.

The first observations of microbial perception and defense responses in plants were described in the early 1970s and several years later Albersheim et al (1978), demonstrated that endogenous plant cell wall polysaccharides produced during pathogen attack, are also able to elicit similar defense responses (Albersheim and Valent, 1978; Schwessinger and Ronald, 2012). Collectively these microbial signaling molecules and plant cell fragments were referred to as exogenous and endogenous elicitors and are now referred to as PAMPs and DAMPs (Boller and Felix, 2009). PAMPS are usually components of microbes such as flagellin and plant components such as xylanase, fungal chitin, lipopolysaccharide, while DAMPs are host-derived signals such as cell wall fragments and peptides (Pel and Pieterse, 2013).

In the last decade a significant number of PRRs required for perception have been identified, including receptors FLS2, EFR and Xa21 required for perception of bacterial peptide PAMPs as well as several receptors, such as LysM and CERK1, required to perceive fungal cell wall components (Segonzac and Zipfel, 2011; Tena et al., 2011). Perception of PAMPs elicits early-defense responses in intact plants and in plant cell cultures: including ion fluxes across the plasma membrane, extracellular alkalinisation of the cell culture medium, transient increases in cytosolic calcium concentration, protein phosphorylation and dephosphorylation through activation of MAPKs, calcium-dependent protein kinases (CDPKs) and protein phosphatases, as well as the production of extracellular reactive oxygen species (ROS) involving plasma membrane-localized NADPH oxidase (Fig 2, (Nicaise et al., 2009; Tena et al., 2011)). Ultimately these signaling cascades modulate sets of transcription factors that regulate defense gene expression leading to the production of anti-microbial

peptides and chemicals. Collectively these early and later defense responses contribute to enhanced basal immunity or PTI.

As a counter measure against innate immunity some virulent pathogens have evolved mechanisms to suppress PTI by releasing pathogen virulence molecules called effectors into the host cell cytosol to target signaling components (Boller and He, 2009; Cui et al., 2009). Plants have evolved a monitoring system that senses the presence of these effector molecules. This second pathogen sensing mechanism involves direct or indirect recognition of these effectors by intracellular receptors and induces effector-triggered immunity (ETI). ETI is often a faster and stronger response than PTI and is associated with localized cell death or a hypersensitive response (HR) (Boller and He, 2009; Dodds and Rathjen, 2010). This more narrow form of innate immune response is genotype specific and depends on the presence of disease resistance (*R*) genes to recognize pathogen effectors, also known as avirulence factors (*Avr*) (He et al., 2006). Both PTI and ETI activation lead to accumulation of secondary defense molecules such as ROS, ET, JA and salicylic acid (SA), that can act locally and systemically to restrict pathogen proliferation (Pieterse et al., 2012).

Flagellin and FLS2 the model PAMP pair to study signal transduction

PAMP-triggered activation of basal immunity is possibly the first active defense mechanism in response to bacterial perception. Towards the end of the 1990s, Felix et al. (Felix et al., 1999) serendipitously discovered eubacterial flagellin as a general elicitor when attempting to study harpin-like activity from *Pseudomonas syringae* pv. *tabaci* in tomato cell cultures. Harpins are bacterial elicitors of a hypersensitive response in plant cells. They were isolated from the fire blight pathogen *Erwinia amylovora* (Wei et al., 1992) and from the bean pathogen *Pseudomonas syringae* pv. *syringae* (He et al., 1993). Flagellin is the protein subunit of the bacterial flagellum and essential for the bacterial motility (Felix et al., 1999; Gomez-Gomez et al., 1999). The N- and C-terminal part of flagellin is highly conserved in a wide range of eubacteria and recognized by the innate immune system of many plant species, therefore an excellent model PAMP.

In Arabidopsis, the 22-amino acid peptide (flg22) which corresponds to the highly conserved amino terminus of flagellin, is sufficient for triggering PAMP-related responses starting from two minutes, including the activation of MAPK cascades and induction of pathogen responsive transcription factors downstream of the LRR receptor kinase FLAGELLIN-SENSING 2 (FLS2) to halt microbial growth (Chisholm et al., 2006; Boller and Felix, 2009). These responses were characterized using whole plants, plant cells and protoplast systems (Asai et al., 2002; Zipfel et al., 2004; Benschop et al., 2007). Flagellin-treated Arabidopsis seedlings did not only display defense-related responses, but also inhibition of root growth, cotyledons and leaves. These growth effects are dose-dependent and result in an overall reduction in fresh weight increase of the seedlings (Gomez-Gomez et al., 1999).

More recently, *in vivo* [^{33}P]phosphate pulse labeling of Arabidopsis cells demonstrated, that flg22 perception is instantaneously followed by the phosphorylation of

the FLS2 and BRI1- associated receptor kinase (BAK1) complex (Schulze et al., 2010). BAK1 is another LRR-RLK that is also involved in the perception of other PAMPs through different receptor complexes as well as required for the regulation of brassinolide signaling (Chinchilla et al., 2009). Interestingly BAK1 phosphorylation is followed by the phosphorylation of a common set of proteins shared in FLS2 and EFR-mediated signaling (Fig. 2, (Chinchilla et al., 2007; Tena et al., 2011)). These include MAPKs and CDPKs as well as BOTRYTIS-INDUCED KINASE1 (BIK1), a receptor-like cytoplasmic kinase, that is rapidly phosphorylated

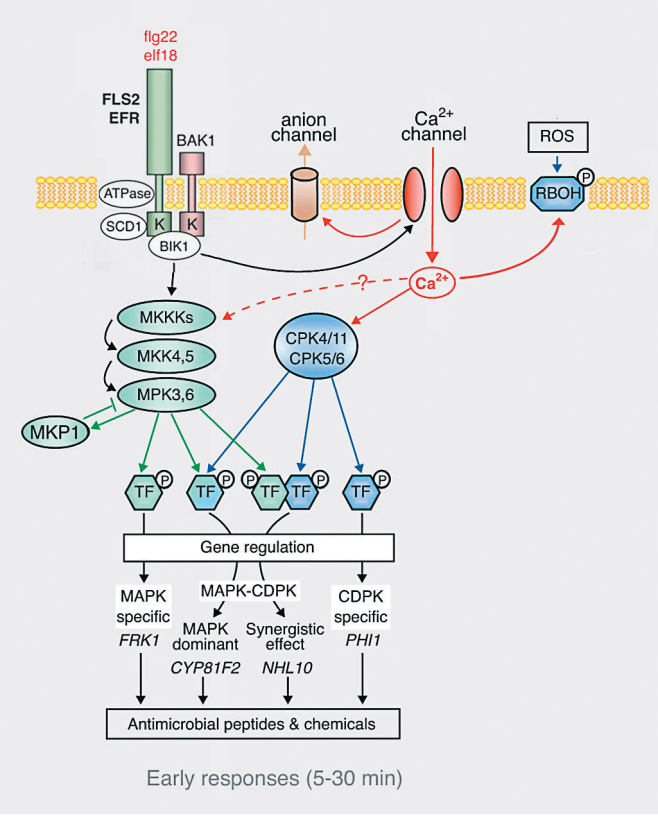

Figure 2. Representation of early PAMP-triggered innate immunity signaling. The leucine-rich repeat (LRR) receptor-like kinase (RLK) FLS2 and EFR (green) perceive the PAMPs flg22 and elf18 (red) and this is immediately followed by the association of BAK1 (pink) with the FLS2 or EFR ligand. BIK1 (white) is phosphorylated and binds to the FLS2-BAK1 or EFR-BAK1 complex followed by the activation of the MAPK module (green). MAPK activity is negatively regulated and controlled by MAPK phosphatase MKP1. The early defense signaling events activate Ca^{2+} influxes, anion effluxes upstream of ROS production and CDPK activation. Both MAPK and CDPK signaling result in phosphorylation of transcription factors (TF), that modulate gene expression of defense-related genes involved in the biosynthesis of antimicrobial chemicals and peptides. Figure adapted from Tena et al. (2011) with permission.

and couples with the heterodimer FLS2-BAK1 (Benschop et al., 2007; Lu et al., 2010). The events immediately downstream of the phosphorylated FLS2/BAK1/BIK1 complex are so far uncharacterized, but via unknown intermediate steps this is followed by the sequential phosphorylation of MAPK cascade members. (Fig. 2, Tena et al., 2011).

The flg22-induced phosphorylation of several MAPK kinase kinases as well as a large set of MAPKs has been demonstrated by (quantitative) phospho-proteomic approaches (Benschop et al., 2007; Nakagami et al., 2010; Mithoe et al., 2012) and these datasets may hold clues to the missing phospho-proteins, linking receptor complexes and downstream MAPK cascades. Nonetheless, our detailed understanding of plants responsiveness to PAMPs and PTI relates largely to the perception of flagellin and has set the standard for most laboratories.

Elongation factor Tu and EFR, a second potent signaling pair

Another elicitor of Arabidopsis PTI is one of the most abundant and conserved bacterial proteins, elongation factor thermo unstable (EF-Tu) (Kunze et al., 2004). The first 18 amino acids of the N-terminus of EF-Tu (elf18) are sufficient to induce plant defense responses in Brassicaceae. Elf18 is perceived through EFR, a PRR that also requires BAK1 as a co-receptor and is transphosphorylated similar to the FLS2-BAK1 receptor complex phosphorylation after treatment with flg22 (Schulze et al., 2010). While the perception of flg22 requires FLS2 (Gomez-Gomez et al., 1999), EF-Tu is still perceived in plants carrying mutations in FLS2 (Kunze et al., 2004). FLS2 and EFR are closely linked LRR-RLKs, that likely diverged during evolution to become more specialized to detect different MAMPs.

While most PTI signaling events tested so far seem to be shared between FLS2 and EFR signaling cascades, including the requirement for BAK1, induction of anion channels (Ca^{2+}) channel activity, reactive oxygen species (ROS) production and MAPK and CDPK activation, as well as BIK1 phosphorylation, some differences in requirements for EFR signaling have been identified. Through a genetic suppressor screen, the EFR receptor was shown to be more dependent on a quality control system during maturation in the ER (Nekrasov et al., 2009). Although EF-Tu and its peptides elf18 and elf26 are potent PAMPs, EFR signaling in the roots of Arabidopsis is not detectable when stimulated with the elf26 peptide (Millet et al., 2010). The same study describes flg22-induced defense gene expression in roots, indicating that FLS2 and EFR signaling may also differ with respect to tissue specificity.

MAPK cascades in innate immunity

MAPK cascades are evolutionarily conserved regulatory modules, which function downstream of cell-surface receptors and transmit extracellular stimuli to critical regulatory targets within cells. They are present in diverse signal transduction pathways of eukaryotic cells from yeast to human and plants. The functionally linked MAPK protein module consists of three subsequently acting protein kinases, a serine (Ser) or threonine (Thr) MAPK kinase kinase (MAPKKK or MEKK), which phosphorylates the conserved Ser/Thr X_{3-5}- Ser/Thr motif

present in the activation loop of the downstream MAPK kinase (MAPKK or MEK). The activated MEK phosphorylates Thr and Tyr amino acid residues in the conserved T-x-Y motif located in the activation loop of MAPKs (Ichimura et al., 2002; Jonak et al., 2002; Rodriguez et al., 2010). Phosphorylation of MAPK cascades leads to transmission and amplification of the transduced signal and subsequently to the phosphorylation of downstream targets, which in plants include WKRY and ERF transcription factors (Menke et al., 2005; Bethke et al., 2009; Mao et al., 2011), as well as factors involved in ET biosynthesis and regulation, such as ACS2 and EIN3 (Liu and Zhang, 2004; Yoo et al., 2008). The deactivation and regulation of MAPK activity is mediated by MAPK phosphatase 1 (MKP1) and protein phosphatase 2C (PP2C1), which function as tyrosine and serine/threonine-specific phosphatases (Schweighofer et al., 2007; Bartels et al., 2009; Bartels et al., 2010).

The genome of Arabidopsis encodes over 80 MAPKKKs, 10 MAPK kinases MAPKKs and 20 MAPKs. Both Arabidopsis MAPKs and MAPKKs are classified into four subclasses, A–D (Ichimura et al., 2002). The subclasses A-C contain a TEY phosphorylation motif in the activation loop and the subclass D contains a TDY motif in the MAPK activation loop. Arabidopsis MAPKs, MPK3 and MPK6 belong to the group A subclass, as are their orthologues in *Nicotiana tabacum* (tobacco), *Medicago sativa* (alfalfa), rice, and poplar. These MAPKs are involved in many signaling cascades leading to diverse responses, including biotic and abiotic stress responses, phytohormonal response, embryonic and stomatal patterning (Zhang and Klessig, 1997, 2000; Menke et al., 2004; Menke et al., 2005; Wang et al., 2007; Rodriguez et al., 2010). Group B MAPKs are related to Arabidopsis MPK4, which is implicated in pathogen defense and abiotic stress responses, cytokinesis and cytoskeletal organization (Petersen et al., 2000; Brodersen et al., 2006; Meszaros et al., 2006; Kosetsu et al., 2010; Beck et al., 2011).

In Arabidopsis, MAPK activation belongs to the earliest PAMP signaling event to occur after flagellin perception by FLS2. In leaf protoplasts a complete MAPK cascade composed of MEKK1, MEK4/5, and MAPK3/6 was identified in response to flg22 stimulation (Asai et al., 2002). This module amplifies and transduces the flagellin signal and ultimately phosphorylates various defense-related transcription co-activators and WRKY transcription factors which play a role in early responses in plant immunity (Rodriguez et al., 2010; Tena et al., 2011). MAPK activation may need to reach a threshold in duration and magnitude to activate secondary and late responses. This threshold is also controlled by MKP1, that down-regulates MPK3/6 activity within 10–15 min after elicitation in protoplasts and leaves (Anderson et al., 2011; Tena et al., 2011). Interestingly, in parallel elicitation by flg22 also activates another MAPK module composed of MEKK1-MKK2/MKK1-MPK4 (Meszaros et al., 2006; Suarez-Rodriguez et al., 2007). MPK4 was previously implicated to negatively regulate systemic acquired resistance (SAR) (Petersen et al., 2000). The balance between positive regulation of MPK3/6 and the negative regulation of MPK4 is likely necessary to tightly control defense responses after pathogen perception.

Interestingly both the MPK4 and the MPK3/6 cascade have been associated with WRKY33. Prior to PTI signaling, MPK4 and WRKY33 form a complex in the nucleus that also

requires the MPK4 target MAP kinase substrate 1 (MKS1) (Qiu et al., 2008). Upon pathogen perception, WRKY33 is released from the complex and activates *phytoalexin deficient 3* (*PAD3*) expression required for camalexin biosynthesis. Phosphorylation of WRKY33 by MPK3 and MPK6 was subsequently shown to be required for activation of camalexin biosynthesis gene expression (Mao et al., 2011). This suggests that, although MPK4 can act as a negative regulator of SAR, flg22-triggered MPK4 activation may act in concert with activated MPK3 and MPK6 to control phytoalexin biosynthesis.

MAPKKKs in basal defense signaling

MAPKKKs are the upstream components of the MAPK phosphorylation cascade involved in transmitting extra- and intracellular signals to downstream protein kinase cascade members. This family comprises the most heterozygous and largest group of MAPK pathway components with 80 members, but relatively little is known about their function in plants (Frye et al., 2001; Jin et al., 2002; Champion et al., 2004). Relationship analysis on the basis of sequence analysis of the protein kinase catalytic domain divides MAPKKKs into two major subtypes: MEKKs and RAF-like kinases (Ichimura et al., 2002). MEKK-like subfamily members have kinase domains similar to mammalian MEKK1 and to yeast STE11 and BCK1, whereas RAF-like kinases are more similar to mammalian RAF1 (Champion et al., 2004).

Only a few members of the MEKK subfamily have been studied in more detail: Arabidopsis MEKK1, in response to flg22 perception, tobacco NPK1 protein, which is involved in innate immunity and cytokinesis (Nishihama et al., 2001; Jin et al., 2002) and tobacco MAPKKKα and tomato MAPKKKε, which are involved in regulating cell death (del Pozo et al., 2004; Melech-Bonfil and Sessa, 2010). Interestingly, MEKK-like members MAP3Kε1 and MAP3Kε2 are involved in cell division and pollen viability and have not been proved to act in MAPK cascades. They are functional homologues of fission yeast Cdc7, which suggests that MAP3Kε1 and MAP3Kε2 may not function as MAPKKKs (Ichimura et al., 2002; Champion et al., 2004; Chaiwongsar et al., 2006).

MEKK1 has been implicated upstream of MKK4/MKK5 and MPK3/MPK6 by transient expression studies in protoplasts (Asai et al., 2002). Controversially, another study found that MEKK1 is required for flg22-induced activation of MPK4, but not for the activation of MPK3 and MPK6 (Suarez-Rodriguez et al., 2007). This same study also showed that MEKK1 kinase activity is not required for flg22-induced MPK4 activation, but that disease resistance of the *mekk1* mutant complemented with the kinase dead version of MEKK1 was compromised. These results may show that MEKK1 functions as a scaffold for the MPK4 pathway involved in negatively regulating SA- mediated defense response (Petersen et al., 2000). It is likely that multiple MAPKKK family members positively act upstream of MKK4/MKK5 and MPK3/MPK6 activation.

The RAF-like MAPKKKs, constitutive triple response 1 (CTR1) and enhanced disease resistance 1 (EDR1) have close homologues in *Physcomitrella* and *Selaginella* and they are involved in ET and disease resistance signaling (Ichimura et al., 2002). CTR1 is a negative

regulator of ethylene response and acts upstream of the transcriptional regulator ethylene-insensitive 3 (EIN3). CTR1 was shown to inhibit the MKK9-MPK3/MPK6 module, which regulates the stability of the EIN3 protein in response to ET (Yoo et al., 2008). The kinase activity of CTR1 is inactivated by ET, however the MAPK cascade components and targets downstream of CTR1 are unknown and the negative regulation of the MKK9-MPK3/MPK6 module was shown by genetics and is likely indirect (Yoo and Sheen, 2008; Bethke et al., 2009; Yoo et al., 2009; Bisson and Groth, 2011).

EDR1 is a related kinase, classified in the same subfamily as CTR1 and a negative regulator of SA-induced defense responses. The *edr1* mutant shows enhanced resistance to bacterial and fungal pathogens (Frye et al., 2001; Tang et al., 2005). Similar to CTR1, the immediate downstream kinase cascade or targets of EDR1 are yet unknown, despite the fact that these kinase were identified many years ago.

MAPKKKs in developmental pathways

MAPK cascades have also been associated with developmental processes and hormonal responses, including auxin responsiveness. Kovtun et al. (1998) used a maize protoplast transient expression system to demonstrate that *Nicotiana* protein kinase 1 (NPK1), a MKKK member, acts as a negative regulator in auxin signal transduction. Furthermore, transgenic tobacco plants overexpressing the NPK1 kinase domain potentially disturb embryogenesis in transgenic tobacco seedlings. Additionally the Arabidopsis orthologue of NPK1-like protein kinase (ANP1) mediated MAPK cascade, demonstrated a molecular link between oxidative stress and the plant growth hormone auxin (Kovtun et al., 2000). NPK1 and its Arabidopsis orthologues ANP1, 2 and 3 have also been connected with cytokinesis and are expressed in highly dividing tissues (Rodriguez et al., 2010). The downstream MKK and MAPK for tobacco as well as for Arabidopsis have been identified. In tobacco NPK1 acts upstream of MKK NQK1 and MAPK NRK1/Ntf6. The corresponding Arabidopsis cascade downstream of ANP1/2/3 consists of MEK6 and MPK4. Ntf6 and MPK4 both localize to the division plane where they phosphorylate the microtubule associate protein MAP65-1 to affect its microtubule binding affinity (Sasabe and Machida, 2006; Sasabe et al., 2006).

The best characterized developmental program regulated by MAPKs is stomata development. Stomatal patterning involves the peptide ligand EPIDERMAL PATTERNING FACTOR 1 (EPF1) and receptor like kinases TOO MANY MOUTHS (TMM) and ERECTA-likes upstream of activation of the YODA, MEK4/5, MPK3/6 pathway (Dong et al., 2010). This MAPK cascade negatively regulates the transcription factor *SPEECHLESS* (Lampard et al., 2008) required for initial division of the meristemoid mother cell (MacAlister et al., 2007), demonstrating that meristematic divisions in leaf epidermal cells are controlled by a functional MAP kinase.

Overall we can conclude that in plants MAPK signaling involves a redundancy of signaling components and intertwining of these protein kinase modules. In order to dissect and understand MAPK signaling, further systematic analysis of the genes involved requires the useof genetic, genomic, and proteomic approaches, as well as *in vivo* functional assays.

SCOPE OF THIS THESIS

The work described in this thesis centers around the rapid responses in plants and plant cells to PAMP-induced post-translational modifications (PTMs). Many signaling cascades are regulated by PTMs, including the MAPK cascade that controls early defense-related responses in plant-pathogen interactions. This phosphorylation cascade has an important role in innate immunity signaling, growth and developmental pathways. Despite extensive research, our understanding of the molecular mechanisms regulating MAPK signaling is hindered by the complexity in signaling pathways. Our research demonstrates that the phosphorylation status of MAPK members determines the regulation of basal immunity and plant development.

Chapter **two** elaborates on the importance of protein phosphorylation in signal transduction in plants with a focus on Tyr phosphorylation. Protein phosphorylation and in particular Tyr phosphorylation, has been technically challenging to measure and an overview of technical issues, such as sample preparation and phosphopeptide enrichment methods is presented. In this context the recent advances on measurement of Tyr phosphorylation in plant signal transduction are described. Furthermore, the contribution of Tyr phosphorylation to plant signal transduction and its relative importance are discussed in relation to other eukaryotic models systems.

Chapter **three** describes a targeted proteomics approach to measure Tyr phosphorylation in plant signal transduction research. In this study metabolic labelling of Arabidopsis cells is combined with an immuno-affinity purification method to target the Tyr phosphopeptide fraction for mass spectrometric identification. This quantitative approach identified a set of 149 phosphorylated Tyr peptides. In addition to the identification of the three main players in flg22-induced MAPK signal transduction as Tyr phosphorylated on the TEY motif, less studied MAPK members were also implicated in flg22 signaling. This study elucidated a new level of complexity in flagellin-induced MAP kinase activation.

In chapter **four** the functional analysis is described of the phosphoprotein MKKK7 for which we uncovered a function in innate immunity. Our work reveals that in the *mkkk7* mutant there is enhanced defense gene activation, which is accompanied by enhanced resistance to the bacterial pathogen *Pseudomonas syringae*. Conversely, complementation by *MKKK7* overexpression resulted in a shutdown of flg22-induced defense responses and an increase in disease susceptibility. Through inducible overexpression of *MKKK7* in plants we demonstrated that MKKK7 acts via suppression of flg22-induced phosphorylation of MAPKs. Furthermore, replacement of the phosphorylated residues in MKKK7 supported the biological relevance of phosphorylation of MKKK7 in repressing basal immunity signaling in Arabidopsis.

Chapter **five** describes our work on the same phosphoprotein MKKK7 in root development. The *mkkk7* knockdown mutant demonstrated a short-root phenotype with associated changes in the root meristem. Overexpression of *MKKK7* resulted in enhanced growth with longer roots and enlarged root meristem phenotypes. We show that this is also

partly dependent on phosphorylation of MKKK7. MKKK7 function is connected to peptide signaling in root meristem maintenance by showing that the *mkkk7* mutant can be rescued after application of tyrosine-sulphated peptides of the RGF family. Therefore this MAPKKK cascade may function in the peptide signaling pathway controlling root development.

Finally the results described in chapter **three, four** and **five** are discussed in chapter **six** with reference to the current knowledge on tyrosine phosphorylation, basal immunity and growth and development in plants.

CHAPTER 2

Phosphoproteomics perspective on plant signal transduction and tyrosine phosphorylation

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ABSTRACT

Plants and animal cells use intricate signaling pathways to respond to a diverse array of stimuli. These stimuli include signals for the environment, such as biotic and abiotic stress signals, as well as cell-to-cell signaling required for pattern formation during development. The transduction of the signal often relies on the post translational modification (PTM) of proteins. Protein phosphorylation in eukaryotic cells is considered to be a central mechanism for regulation and cellular signaling. The classic view is that phosphorylation of Serine (Ser) and Threonine (Thr) residues in more abundant, whereas Tyrosine (Tyr) phosphorylation is less frequent. This review provides an overview of the progress in the plant phosphoproteomics field and how this progress has led to a re-evaluation of the relative contribution of tyrosine phosphorylation to the plant phosphoproteome. In relation to this newly appreciated contribution of tyrosine phosphorylation we also discuss some of the recent progress on the role of tyrosine phosphorylation in plant signal transduction.

INTRODUCTION

Plant signal transduction

Extracellular signals are perceived by cells through plasma membrane (PM) receptors that transduce the signals to an intracellular signal transduction cascade that ends in the activation of transcription of the appropriate set of genes. The transduction of the signal relies in most cases on posttranslational modification of the signaling proteins and the generation of so called second messenger molecules. The best-studied and understood posttranslational modification is protein phosphorylation, which can lead to changes in conformation, protein-protein interaction and protein activity. In eukaryotic cells protein phosphorylation occurs predominantly on serine, threonine and tyrosine residues, but has also been described to occur on aspartate en histidine residues.

Over the last decade the field of plant signal transduction has seen a tremendous development. The analysis of protein phosphorylation has gone from protein by protein basis to high through put analysis of phosphorylation at a proteome scale. Protein phosphorylation occurs predominantly on Ser, Thr and Tyr residues which contain a hydroxyl group that can accept a phosphoryl group. This reversible reaction is catalyzed by protein kinases. Protein dephosphorylation is catalyzed by protein phosphatases. Plants have an extensive family of protein kinases and protein phosphatases, with nearly 1000 genes encoding kinases in Arabidopsis and 1467 genes in rice and 300 and 132 genes encoding protein phosphatases in Arabidopsis and rice respectively (Arabidopsis Genome Initiative, 2000; Kerk et al., 2006; Dardick et al., 2007; Jung et al., 2010; Singh et al., 2010). The combined activity of kinases and phosphatases, which are in dynamic equilibrium, determines the level of phosphorylation for individual substrate proteins. Moreover, kinases and phosphatases are frequently regulated by negative feedback loops that attenuate the signaling back to basal levels (Brandman and Meyer, 2008). Initial efforts to study protein phosphorylation in plant signal transduction made use of a pharmacological approach to inhibit protein kinases and phosphatases. The mostly general inhibitors, such as staurosporine and K252a, could be used to show a role for protein kinase activity in stimulus-induced responses. Using this approach elicitor induced ion fluxes across the plasma membrane, generation of second messengers, defense gene expression and phytoalexin production were shown to require protein phosphorylation (Scheel, 1998; Menke et al., 1999). This was complemented by identification and cloning of specific kinases that played a role in plant defense responses and disease resistance (Martin et al., 1993; Song et al., 1995; Zhang and Klessig, 1997). At the turn of the century individual research effort were mostly geared towards identification of protein kinases /phosphatase and the stimuli that induced their activation (Zhang et al., 1998; Romeis et al., 1999; Kovtun et al., 2000; Nuhse et al., 2000; Asai et al., 2002). Reverse genetic approaches based on RNAi and virus induced gene silencing (VIGS) allowed the functional analysis of kinases and phosphatases (Romeis et al., 2001; Jin et al., 2002; Menke et al., 2004), but identification of substrates and specific sites of phosphorylation were still scarce. The available methods

were limited to biochemical approaches, such as solid state phosphorylation screens to identify corresponding substrates or Y2H analysis to identify interacting partners of protein kinases and phosphatases (Qiu et al., 2008). The kinase-dependent phosphorylation of the potential substrates was tested in vitro with recombinant or purified kinase substrate pairs (Menke et al., 2005).

PLANT PHOSPHOPROTEOMICS

Towards plant phosphoproteomics

A first real step towards larger scale phosphoproteomic approaches was the combined use of 2-dimensional gel electrophoresis (2-DE) and spot identification by mass spectrometry. This approach initially made use of radiolabeled protein samples obtained by pulse chase labeling of cells with ^{32}P-orthophosphate (Peck et al., 2001). Detection and identification of phosphorylated peptides was however hampered by the reduced effectiveness of ionization of phosphorylated peptides in complex peptide mixtures in mass spectrometers. Never the less *in vivo* phosphorylated proteins were identified allowing the functional analysis of these phosphoproteins and eventually connecting to the upstream protein kinase (Merkouropoulos et al., 2008).

Phosphopeptide enrichment

The first major step forward towards phosphoproteomics came with the development of enrichment strategies for phosphorylated peptides. Even though about a third of all proteins can be phosphorylated at any time in the cell, phosphorylated proteins are relatively low in abundance. Moreover phosphoproteins with regulatory functions and involved in signal transduction are present at sub-stoichiometric levels and are transient in nature. Phosphorylation events on signaling proteins are highly regulated by the conserted action of protein kinases and protein phosphatases. A small change in stoichiometry between non-phosphorylated and the phosphorylated version of individual signaling proteins can have very significant consequences for the signaling output. These transient and small changes in phosphorylation status thus further hampers the identification of phosphorylated proteins and peptides in complex protein or peptide mixtures. Furthermore, ionization of phosphopeptides (phops) in mixtures with non-phosphopeptides is suppressed and limits their selection and fragmentation in mass spectrometers. Thus enrichment of phosphopeptides from complex mixtures was a prerequisite to the successful large-scale identification by mass spectrometry. Therefore achieving phosphopeptide enrichment was a significant step forward and made it possible to not only identify *in vivo* phosphorylated proteins on a larger scale, but also to confidently identify the residue that was modified (Ficarro et al., 2002; Blagoev et al., 2003; Nuhse et al., 2003; Pinkse et al., 2004).The most commonly used enrichment methods are based on affinity purification of phosphoryl-group

containing peptides and include immobilized metal affinity chromatography (IMAC), strong cation exchange chromatography (SCX) and metal oxide affinity chromatography (MOAC). IMAC uses metal ions such as Fe(III) or Ga(III) immobilized by chelation on a solid substrate. When used at a low pH these positively charged ions can interact selectively with negatively charged phosphoryl groups. After washing under low pH conditions, selectively bound phops are eluted from the IMAC columns using a buffer with a more alkaline pH. Ficarro and collegues were the first to report on the use of IMAC to enrich complex tryptic digestion mixtures for phops and analyzed the enriched fraction by nanoflow HPLC/electrospray ionization mass spectrometry (nano-LC-ESI-MS). They applied this method to characterize the phosphoproteome of whole lysate of yeast cell and identified 383 phosphorylation sites on 216 phops. A large proportion of the identified phops were multi-phosphorylated peptides, and singly phosphorylated peptides were underrepresented. The preferential enrichment of multi-phosphorylated peptides over singly phosphorylated peptides by IMAC was noted in several independent studies. A modified and refined IMAC strategy was used by Nuhse et al. (2003) who analyzed Arabidopsis suspension cultured cells treated with the peptide elicitor flg22. In addition to IMAC they used strong anion exchange (SAX) fractionation to reduce the complexity of the tryptic digest prior to IMAC enrichment. This resulted in a larger proportion of singly phosphorylated peptides in addition to the multi-phosphorylated peptides. With this combined approach the authors reported enrichment up to 75% in phops. In a follow-up study the authors reported a total of 300 phosphorylated sites on 200 phops from plasma membrane associated protein, most of which were newly identified (Nuhse et al., 2004). A drawback of IMAC is the negatively charged carboxyl groups present in peptides that compete with the phosphoryl groups for binding to the metal ions. However, at low pH carboxyl groups are less likely to carry a negative charge and these conditions are more selective for phosphoryl group affinity interactions. A popular and successful alternative to IMAC is metal oxided based affinity purification of phops. Metal oxide (TiO_2 or ZiO_2) columns also have a tendency to bind both phosphoryl and carboxyl groups but by loading the peptides on the column in the presence of acids, such as dihydroxybenzoic acid of lactic acid, the phosphoryl affinity is enhanced. MOAC is also more selective towards mono-phosphorylated peptides (Pinkse et al., 2004; Larsen et al., 2005). Metal oxide purification of phops was successfully used in a number of studies involving plant phosphoproteomics approaches (Benschop et al., 2007; Kersten et al., 2009; Stulemeijer et al., 2009). Benschop et al. (2007), used the metal oxide TiO_2 in combination with strong cation exchange (SCX) to enrich phops from tryptic digests of plasma membrane associate proteins of Arabidopsis. With this combined approach 1172 phops were succesfully identified, of which the vast majority were singly phosphorylated peptides.

Comparative analysis of modified and improved IMAC and TiO_2 methods revealed a significant bias of each method for a non-overlapping portion of the phosphoproteome (Thingholm et al., 2009). This and other comparative studies (Ndassa et al., 2006; Bodenmiller et al., 2007) made it clear that in order to obtain a more complete phosphoproteomic analysis IMAC and TiO_2 based methods should be combined by using them sequentially or

in parallel on the same samples. In addition to the methods described above, additional methods, both commercial and in house developed, and modifications to existing protocols have been described. For further in dept reading on these subjects we recommend reviews by Kersten et al. (2006; 2009) and Thingholm et al. (2009).

Separation and detection of phosphoproteins and phosphopeptides

Sample preparation and enrichment of phosphopeptides are two important steps in succesful proteomics experiments which are then followed by one or two dimensional separation of the protein/peptide mixture prior to mass spectrometric identification. Broadly speaking these separation techniques can be divided into gel-based seperation of proteins, such as one and two dimensional gel electrophoresis, and chromatography based peptide separation such as strong cation/anion exchange followed by (online) reverse phase liquid chromatography. One and two dimensional gel electrophoresis (1- or 2-DE) have long been the method of choice to separate protein mixtures into discrete bands of protein or protein spots. Protein bands in 1-DE often still consist of multiple proteins and phosphorylated forms of proteins often cause only minor shifts in the electrophoretic mobility as compared to the non-phosphorylated version. In 2-DE proteins are separated on charge by isoelectric focusing as well as on molecular weight in the second dimension, resulting in highly complex patterns of proteins spots, which mostly consist of individual proteins. Furthermore, phosphorylated proteins give rise to the so called 'string of pearls' patterns in which each spots represents a different number of phosphorylated residues in the protein isoform, as recently shown for the brassinolide-regulated change in phosphorylation of BRZ1 (Tang et al., 2008a). During the first dimensional separation different hyperphosphorylated BZR1 forms migrated to a position at the acidic end of the gel whereas different hypophosphorylated forms of BZR1 migrated to positions in the gel with a more basic pH. Since the phosphorylation status has little effect on migration in the second dimension the different phosphorylated forms of BZR1 gave rise to a row of spots (Tang et al., 2008a), resembling a 'string of pearls'. Visualization of phosphoproteins in gel based separation techniques is essential for subsequent isolation and identification of the protein by mass spectrometry. Initially this was achieved by pulse chase labeling of cells with radioactive phosphate (^{32}P-orthophosphate) and autoradiography of gel, which was followed by excision of the correct spots from analytical gels (Peck et al., 2001). Radioactive labeling has since then become more or less obsolete with the development of the phosphoprotein staining dye ProQ diamond which allows detection of phosphoproteins through fluorescent laser scanning (Jin et al., 2006). This method is compatible with concomitant total protein staining with Sypro Ruby stain with fluoresces with a different spectrum. A third and quantitative method of labeling uses covalently linked Cy dyes that are cross-linked to protein samples prior to separation. Up to three Cy dyes (Cy2, Cy3 and Cy5) each with different excitation and emission spectra can be simultaneously used (Tonge et al., 2001; Tannu and Hemby, 2006; Tang et al., 2008a; Tang et al., 2008b). This allows different conditions to be compared on a single 2-DE gel. This circumvents the running and

comparison of individual gels with concomitant technical variation. Following separation and isolation of protein spots, which can be done automated with the use of robotics, the proteins are identified of Maldi-TOF or Q-TOF mass spectrometers. An in depth overview of different types of mass spectrometers and their usage can be found in Boersema et al. (2009a).

Chromatography based separation is mostly done on peptide mixtures and uses two qualities, charge state of the peptide and its hydrophobicity. The most commonly used method that is based on charge state affininty purification for phosphoproteomics applications is strong cation exchange (SCX). Fractionation of peptide mixtures by SCX is based on the interaction of positively charged groups on the peptides with the negatively charged groups in the SCX resin. Peptides are loaded onto the column under low salt low pH conditions and eluted by an increasing salt gradient. The additional negative charge on phosphopeptides causes a weaker interaction and elution under lower salt conditions as compared to the same peptides without the phosphoryl group. The initial fractions from SCX are thus enriched in phops while later fractions are almost completely depleted of phops. Many studies have used SCX to prefractionate phosphopeptide mixtures into deconvoluted fractions that are than further enriched for phopsphopeptides by IMAC or MOAC (Table I).

The enriched phosphopeptide fractions are finally further fractionated by reverse phase LC, which separates peptides based on hydrophobicity. The reverse phase LC fractionation is done online with the mass spectrometer and is usually referred to as ESI-nano-LC-MS, where ESI stands for electro spray ionization and nano denotes the elution in nanoliter per second. Elution from the nano-LC goes straight into the ionization chamber where the eluting droplets are vaporized and ionized by high voltage. The ionized peptides are then accelerated into MS by a powerful electric field. In this first stage MS the peptide ions are separated by mass charge (m/z) ratios and the m/z ratios are recorded in a so called survey scan. The mass spectrometer then selects the most abundant peptide ions for tandem MS using data dependent analysis. The selected peptide ion, or precursor ion, is subjected to fragmentation, usually by collision activated dissociation (CAD). The resultant fragment ions are recorded in a product-ion scan. Because each survey scan contains multiple precursor ions, several product ion scans are done for each survey scan. This is possible because the cycle of survey and product-ion scans is completed very fast in comparison to the elution time of the peptides. The resulting product-ion spectra are recorded and matched to the precursor ion m/z value. During seperate data analysis this information is used to deduce the peptide amino acid sequence and detect possibile post-translational modifications. Most commonly used mass spectrometers in tandem MS phosphoproteomics are, Ion traps, LTQ-Orbitraps and Fournier-Transform ion cyclotron resonance mass spetrometers (FT-ICR-MS). These mass spectrometers have been developed over the last decade and can now operate at sub-femtomolar detection and with low ppm mass accuracy.

For an overview of mass spectrometers and fragmentation methods the reader is referred to reviews by (Boersema et al., 2009a; Schulze, 2010). The rapid development of enhanced sensitivity and increase mass accuracy has made it possible to obtain larger

Table I. Overview of plant phosphoproteome studies using shotgun MS approaches.

Species	Tissue	Treatment	Enrichment	Quantitation	Main features	Reference
Arabidopsis	Cell culture	Phyto-hormone	TiO_2	Label free	Quantitative temporal analysis of 5 phytohormone treatments, 152 differential phops on 136 proteins.	(Chen et al., 2010)
Arabidopsis and rice	Cultured cells	Untreated	SCX and TiO_2	None	6,919 phosphopeptides from 3,393 proteins from rice showing significant levels of Tyr phops. Conservation of phosphorylation of orthologues in three species	(Nakagami et al., 2010)
Medicago	Root	Untreated	SCX and IMAC	None	Use of CAD and ETD fragmentation,3,457 unique phosphopeptides from 829 proteins, significant Tyr phosphorylation	(Grimsrud et al., 2010)
Arabidopsis	Leave and shoot	Light treatment	SCX, IMAC and TiO_2	None	3,029 unique phosphopeptides from 1,429 phosphoproteins detected, focus on chloroplast phosphoproteins and their cognate kinases	(Reiland et al., 2009)
Tomato	Seedling	Hypersensitive response	TiO_2	Label free	50 phosphoproteins identified with 12 differential for HR. Photosynthetic activity is specifically suppressed in a phosphorylation-dependent way during the very early stages of HR development.	(Stulemeijer et al., 2009)
Arabidopsis	Seedlings and protoplasts	Untreated	SCX and TiO_2	None	identified 416 phosphopeptides from 345 proteins, used sub-cellular fractionation to to obtain substancial enrichment of nuclear phosphoproteins	(Jones et al., 2009)
Arabidopsis	Cultured cells	Untreated	TiO_2 IMAC ZrO_2	None	Identified 2597 phosphopeptides from 1346 proteins. First report on pS/pT/pY distribution (85.0, 10.7, and 4.3%) similar to animals systems	(Sugiyama et al., 2008)

Species	Tissue	Treatment	Enrichment	Quantitation	Main features	Reference
Arabidopsis	Cultured cells	untreated	IMAC	None	Identification of 303 phosphorylation sites on 205 proteins. Reported pS/pT/pY ratio of 91.8%/7.5%/0.7%.	(de la Fuente van Bentem et al., 2008)
Arabidopsis	Cultured cells	Sucrose	IMAC	Label free	Temporal study of sucrose resupply. 67 phosphopeptides identified, quantified over five time points	(Niittyla et al., 2007)
Arabidopsis	Cultured cells	Elicitor	SCX and TiO_2	15N metabolic labeling	Quantification based of metabolic labeling. 1172 phops from 472 protein, 98 differential phops from 76 proteins. Highly complex differential phosphorylation of individual proteins.	(Benschop et al., 2007)
Arabidopsis	Cultured cells	Elicitor	IMAC and SCX	iTrac	Quantitative temporal analysis of flg22 signaling using iTrac. 279 phops identified, 11 differential phops on 8 PM proteins. Relevance of phosphorylation for RbohD activity shown.	(Nuhse et al., 2007)
Arabidopsis	Cultured cells	Elicitor	IMAC and SCX	None	First large scale phosphoproteomics study in plants, identified 300 phosphorylation sites in 283 phops. Includes motif analysis and phospho site conservation in orthologues	(Nuhse et al., 2004)

amounts of phosphoproteomic data from more complex samples but also increasingly smaller amount of proteins. This opens up the measurement of more diverse samples than subcellular fractionated suspension cultured cells which up to recently have been the main model system for many plant studies (Table I). These developments have also contributed to the more comprehensive analysis of phospho-proteome samples. However despite the enhanced performance of state-of-the-art mass spectrometers consecutive analysis of the samples still shows only partial overlap between the replicate datasets (Schulze, 2010).

Quantification of phosphorylation

Identification of phosphorylated residues on proteins is only the first step in understanding signal transduction based on changes in phosphorylation. Quantification of changes in protein phosphorylation in response to specific stimuli is required to characterize the

relationship between the phosphorylation of specific residues in a protein and how it affects the proteins behavior. To this end a number of ways to quantify changes in protein phosphorylation have been developed (Fig.1 and Table I). In 2DE based phosphoproteomics the aforementioned labeling with different Cy dyes allows for the quantification of the changes in protein phosphorylation using difference gel electrophoresis (DiGE) (Tang et al., 2008a). By labeling the mock treated sample with Cy3 dye and the brassinolide stimulus induced sample with Cy5 the authors were able to mix and run the two protein samples on one gel. Upon scanning the electrophorized gel for each fluorophore independently and then applying false color imaging and overlaying these false colored images changes in protein phosphorylation can be visualized and quantified in a manner very similar to that used in 2-color DNA microarrays (Tang et al., 2008a).

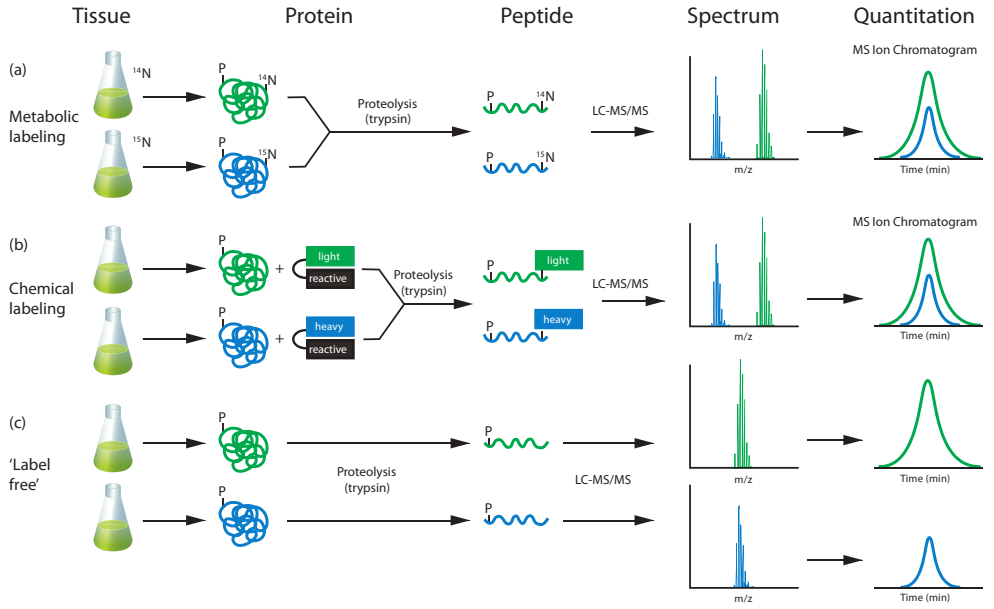

Figure 1. Quantitative approaches used for plant phospho-proteomics. **A**) Metabolic labeling uses 14N and 15N nitrate and ammonium salt in plant growth media to obtain stable isotope labeled proteins. Light and heavy protein samples are mixed prior to digestion and analyzed by LC-MS/MS. **B)** In chemical labeling proteins (or peptides) are labeled after extraction with a light or heavy isotope tags after which the isotope tag proteins are mixed and digested prior to LC-MS/MS analysis. In metabolic labeling and chemical labeling light- and heavy-labeled peptides show different m/z ratios during MS, which allows their relative quantification based on the extracted MS ion chromatogram of the elution of corresponding peptides. **C)** In label-free quantitation samples are extracted and processed separately including the LC-MS/MS analysis. Alignment of MS ion chromatograms is essential prior to quantification.

In 2D DiGE based proteomics methods quantification and MS analysis are performed separately. Other quantification protocols rely on identification and quantification in coupled

MS and MS-MS runs (Fig. 1). Several methods for quantification including dimethyl labeling, iTRAQ, SILAC and $^{14}N/^{15}N$ metabolic labeling as well as label-free quantification have been described (Gruhler et al., 2005; Benschop et al., 2007; Nuhse et al., 2007; Boersema et al., 2009b; Stulemeijer et al., 2009). The first two methods rely on post extraction chemical labeling of the peptide digests with tags with different stable isotopes (Fig. 1b). Isobaric tags for relative quantification (iTRAQ) labels the peptide digest on lysine residues with one of several available isobaric tags that are behaving chromatographically identical. These isobaric tags are composed of different combinations of stable isotopes and yield reporter ions upon collision-induced dissociation (CID) in the mass spectrometer that can be used for identification and quantification. Since 8 different isobaric tags are available up to 8 samples can be multiplexed and analyzed in a single MS run allowing for easy quantification of e.g. time course experiments. This method was used to analyze a time course experiment of flg22 treated suspension cultured cells to identify divergent dynamics of different phosphorylation sites within individual PM H+-ATPases as well as coordinate regulation of conserved phosphorylated residues in homologuous proteins (Nuhse et al., 2007). Another chemical labeling method that is widely used is dimethyl labeling which has been recently been modified to allow multiplexing of three samples (Boersema et al., 2009b). Although this method is fast and inexpensive and does not require measurements in a low m/z range (a disadvantage of iTRAQ) it has not been reported yet for use in plant quantitiative proteomics studies. The most widely used method in quantitative phosphoproteomic experiments is stable isotope labeling with amino acids in cell culture (SILAC). However this method has limited applicability in plants and plant cell cultures due to the fact that plant cells can synthesize the amino acid most commonly used for this type of metabolic labeling experiments and thus only a 70% labeling efficiency can be obtained, which makes quantification difficult (Gruhler et al., 2005). However another metabolic labeling method using ^{15}N nitrate and ammonium salts (Fig. 1a) has been successfully implemented for use in plant cells by Benschop et al. (2007). $^{14}N/^{15}N$ metabolically labeled Arabidopsis suspension cultured cells were used for quantitative phosphoproteomics experiments to identify differentially phosphorylated proteins in response to two different elicitors. By using biological replicate samples that were inversely labeled the authors obtained a high confidence set of 96 differentially phosphorylated peptides from 76 proteins. In addition, a large set of differential phops that were observed in only one of the biological replicates was reported. The results demonstrated an important role for vesicle trafficking in response to elicitor induced defense signaling and plant defense response. Interestingly, it also pointed out the highly complex pattern of phosphorylation of the RbohD protein in response to the two different elicitors. RbohD is one of the subunits of NADPH oxidase in Arabidopsis that generates reactive oxygen species in response to stress. Several of the same phosphorylation events were subsequently also reported by Nuhse et al. (2007) and shown to be required for the activation of RbohD. ^{15}N metabolic labeling can also be applied to seedlings and whole plants for the quantitative proteomic analysis of in planta processes, such as proteomic changes in response to oxidative stress in leaf tissue (Bindschedler et al.,

2008). MS based quantification of changes protein phosphorylation has also been reported without the use of stable isotopes (Fig. 1c). In this approach, called label-free quantitation, the phosphopeptides are quantified based on MS ion peak area. This approach requires the alignment and calibration of chromatograms of the samples that are compared and requires the accurate determination of mass deviation and retention time variation. Using this approach changes in protein phosphorylation were analysed in tomato plants mounting a hypersensitive response (HR) (Stulemeijer et al., 2009). Using TiO_2 and nano-LC-MS/MS 50 phosphopeptides were identified of which 12 peptides showed changes in abundance upon HR initiation. The results suggest that during initial mounting of an HR photosynthetic activity is suppressed in a phosphorylation-dependent manner. Label free quantitation has also been successfully used in time course experiments to identify changes in phosphorylation in response to sucrose sensing (Niittyla et al., 2007). In this report differentially phosphorylated residues were identified in PM H+-ATPases that are required for proton pumping activity in response to sucrose feeding. Further in depth reading on quantitative proteomics approaches can be found in Schulze and Usadel (2010).

From phosphoproteomic data sets to signal transduction

The technical progress of recent years has been accompanied with an increasing number of large-scale phosphoproteomics data sets, several of which contain quantitative data and some of which also include temporal profiling data (Table I). Several of the studies reporting these large-scale phosphoproteomic data sets have been discussed in the previous sections and a more complete overview can be found in other reviews on the subject of plant phosphoproteomics (de la Fuente van Bentem and Hirt, 2007; Thelen and Peck, 2007; Kersten et al., 2009; Schulze, 2010). Although large phosphoproteomic data sets are a first step towards understanding plant signal transduction the challenge is to filter those phosphorylation events that are modulated under specific conditions and contribute to the transduction of the perceived signal. There are several ways to approach this challenge, which include generation of quantitative data sets, temporal profiling experiments as well as in depth bioinformatic analysis of the data. A number of large scale quantitative phosphoproteomic data sets have been generated (Benschop et al., 2007; Niittyla et al., 2007; Nuhse et al., 2007; Stulemeijer et al., 2009; Chen et al., 2010) and each of these have identified specific phosphoproteins and processes modulated in response to the applied stimulus. A number of these identified phosphoproteins, including RbohD and PM H^+ATPases, have been studied in detail in follow up functional analysis and their differential phospho-sites were shown to be required for the modulation of protein activity, as predicted by the initial phosphoproteomic analysis. However, these verifications have to be done on a case by case basis and will take many individual follow up studies to piece together a signal transduction pathway, let alone a complex interconnected signaling network. This is where temporal profiling, preferably in conjunction with relative quantification, will make a difference. Temporal profiling of sufficiently large numbers of phosphoproteins will allow

for the inference of signaling pathways and in due time of signaling networks. This requires significantly more data than currently available in the field of plant phosphoproteomics, but in the animal field this type of analysis has already been successfully implemented (Lemeer and Heck, 2009; Choudhary and Mann, 2010). By implementing computational approaches that bring together coexpression data, protein-protein interactions and subcellular localization with protein phosphorylation data, a predictive kinase substrate signaling network was build that identified kinase substrate pair in DNA damage signaling in human cells (Linding et al., 2007). In time, with more temporal quantitative phosphoproteomic data sets this type of computational analysis will also become within reach of the plant phosphoproteomic field.

Plant phosphoproteomic databases

The availability of these large-scale data sets has led to the creation of a number of phosphoproteomic databases where the data set can be queried and compared. The largest of these is PhosPhAt (http://phosphat.mpimpgolm.mpg.de/) (Heazlewood et al., 2008) which can be queried using Arabidopsis locus identifiers and reports measured and predicted phosphorylation sites for each protein. The computational predictions are trained on the experimentally verified sites contained in the PhosPhAT database. The PhosPhAt database currently contains data on 5170 Arabidopsis phosphoproteins and 32601 phosphosites. The majority of the phosphosites are validated (21.353) whereas the total number of ambiguous phosphosites is 11.248. Out of the total number of phosphosites 12.457 are reported to be unique, indicating that a substantial number of phosphosites have been identified multiple times. The percentage of validated unique pSer in the database is about 76%, pThr represents 17% and pTyr represents 6% of the total validated unique phosphosites. The Plant Phosphoproteome Database (http://phosphoproteome.psc.database.riken.jp) is based on two data sets from the Shirasu lab (Sugiyama et al., 2008; Nakagami et al., 2010) and currently contains data on 5,143 rice phops and 6,919 Arabidopsis phops. The reported ratios of pSer/pThr/pTyr in these two datasets are 84.8%/12.3%/2.9% for rice and 82.7%/13.1%/4.2% for Arabidopsis. A recent addition is the Medicago phosphoproteomics database (http://www.phospho.medicago.wisc.edu) (Grimsrud et al., 2010), which currently contains data of one phosphoproteomics data set from Medicago. This database can be queried based on protein sequence, phosphorylation motif and description and contains 3457 unique phops and shows a pSer/pThr/pTyr distribution of 86%/12.7%/1.3%. An additional resource that is not a phosphoproteomics database persé, but that brings together a wealth of data on proteins kinases and protein phosphatase from many plant species is PlantP (http://plantsp.genomics.purdue.edu/html/).

When considering the data available from plant phosphoproteomic studies and databases the amount of data on phosphorylation on Ser or Thr residues is much more extensive and much fewer phosphorylations on Tyr residues are reported. The reported numbers are very similar to those observed in yeast and animal studies, indicating that

overall the phosphorylation machinery is conserved since an early point in eukaryotic evolution. Despite the relatively limited number of pTyr residues reported this specific modification has gained attention in both the animal and plant signal transduction fields. In the next section we will discuss the available data on Tyr phosphorylation in plant systems and put it in perspective in relation to Tyr phosphorylation in animal systems.

Phospho-tyrosine signaling in plants

There are a number of examples of plant proteins that show phosphorylation on Tyr residues that have been shown to be required for some of the proteins function in vivo. The BRI receptor is one of these proteins, although it was initially characterized as an autophosphorylating serine/threonine kinase. Using anti-phosphotyrosine antibodies it was recently shown that BRI is also phosphorylated on tyrosine residues (Oh et al., 2009b). BRI1 is a PM localized receptor kinase that binds the phytohormone brassinolide (BL) in a complex with its coreceptor BAK1. BL perception requires active BRI1 and activates a phosphorylation-dependent signaling cascade, which was the first cascade to be fully characterized in plants (Tang et al., 2008a; Tang et al., 2008b; Kim and Wang, 2010). Tyrosine phosphorylation on 4 C-terminal residues of BRI1 is important for its kinase activity, whereas phosphorylation of Tyr831 in the juxtamembrane domain causes inhibition of growth and delay in flowering (Oh et al., 2009a). In addition to BRI1, BAK1 and it paralogue BKK1 were also shown to be phosphorylated on tyrosine residues, although the functionality of these events was not shown. However other RLKs, including FLS2, which also forms a complex with BAK1 in flg22 signaling cascade, were also tested in the same experimental set up and did not show Tyr phosphorylation. This demonstrates that Tyr phosphorylation is not a general aspect of RLK signaling in plants. Tyrosine phosphorylation also plays in important role downstream of BRI as it controls the proteasomal degradation of the negatively regulating kinase BIN2, mediated through protein phosphatase BSU1. BIN2 is a GSK3 kinase and autophosphorylates on Tyr200 that is required for kinase activity. This pTyr-dependent activation mechanism is also conserved in mammalian GSK3s. The role of BIN2 in BL signaling is similar to the role played by GSK3ß in Wnt signaling in animal cells. Both kinases phosphorylate their respective targets, BZR1/2 and ß-catenin respectively, to promote their degradation in the cytosol (Kim and Wang, 2010). Another evolutionary conserved family of protein kinases that require tyrosine phosphorylation for their activation are mitogen activated protein (MAP) kinases. MAP kinases act as part of a three tiered cascade containing MAPK kinases and MAPKK kinases phosphorylating each other. MAP kinases are involved in many signaling cascades in plants leading to diverse responses, including biotic and abiotic stress response, phytohormonal response, embryonic and stomatal patterning, cytokinesis and cytoskeletal organization. (Pitzschke et al., 2009; Andreasson and Ellis, 2010). MAP kinases become phosphorylated on the T-x-Y motive of their activation loop by their upstream MAPKK, which can be functionally classified as dual specificity protein kinases (DSK). Reversal of this

dual phosphorylation is required to inactivate the MAP kinases and this achieved by dual specificity MAPK phosphatases (Humberto et al., 2005; Bartels et al., 2010).

All of the above mentioned kinases can be functionally classified as DSKs as they can phosphorylate Tyr residues as well as Thr or Ser residues (Oh et al., 2009a; Oh et al., 2009b). However tyrosine-specific kinases (TSKs) are conspicuously low abundant in the annotated genomes of fully sequenced plant species, with 2 putative TSKs predicted in Arabidopsis and 6 or 7 putative TSKs in Rice (Miranda-Saavedra and Barton, 2007). Furthermore, tyrosine receptor kinases (TRKs), that play a prominent role in receptor mediated signaling in metazoan systems, are completely absent in plant genomes (Initiative, 2000). Interestingly, plants do have an extended family of receptor like kinases (RLK) but these are distinct from TRKs as defined in metazoans, and only a handful of plant RLKs are phosphorylated on tyrosine residue so far. Plants do have some Tyr-specific phosphatases (PTPs) and several dual specificity protein phosphatases (DSPs) (Luan, 2002; Moorhead et al., 2009), but the PTP family in metazoans is much more extensive and seems to have expanded specifically in this evolutionary branch.

pTyr in phosphoproteomic datasets

In non-plant model systems several large scale phosphoproteomic studies have reported levels of serine, threonine and tyrosine phosphorylation to be around 80-85%, 10-15% and 2% respectively (Olsen et al., 2006; Swaney et al., 2009). Initial phosphoprotemics approaches in plants however only showed minor numbers of phosphopeptides containing phosphorylated tyrosine residues (Nuhse et al., 2004; Benschop et al., 2007; Nuhse et al., 2007). This suggested that tyrosine phosphorylation was not as prevalent in plant systems as it was in animal systems. This suggestion was reinforced by the notion that model plant species Arabidopsis and rice have very few TSKs, no TRKs and only limited numbers of DSKs. The first indication that tyrosine phosphorylation may be more abundant then hitherto acknowledged came with the large-scale phosphoproteomic study by Sugiyama et al (2008) that reported 4% Tyr phosphorylation in Arabidopsis cells. In their study, and follow up study by the same group, much more pTyr containing peptides were identified from both Arabidopsis cell cultured cells and rice cell cultured cells (Nakagami et al., 2010). The total number of pTyr containing peptides as well as their relative contribution to the plant phosphoproteome were much more significant then reported before in phosphoproteomic studies by three independent labs. In the later three cases only very few pTyr containing peptides were identified in relatively larger scale studies that ranged from a few hundred (Nuhse et al., 2004; de la Fuente van Bentem et al., 2008) to well over a thousand phosphopeptides (Benschop et al., 2007). Re-evaluation of the data contained in the Sugiyama study suggested that ambiguous phospho-site assignment may have contributed to an overestimation of the number of true Tyr phosphorylation sites, but it also indicated that most phosphotyrosine sites in mono-phosphorylated peptides were correct (de la Fuente van Bentem and Hirt, 2009). Nakagami et al. (2010) also reanalyzed their data using

PTM score-derived localization probability method (Olsen et al., 2006) resulting in adjusted levels of tyrosine phosphorylation of 1.6% for rice and 2.4 % for Arabidopsis. Similar levels of tyrosine phosphorylation were recently also reported in a Medicago phosphoproteomics study on root tissue (Grimsrud et al., 2010). Whereas all previous plant phosphoproteomics studies have used collision-activated dissociation (CAD) during MS/MS to obtain ion fragments from which to deduce peptide sequence information, Grimsrud and colleagues have also used electron-transfer dissociation (ETD) during MS/MS. With CAD the energy to induce dissociation can often result in the preferential loss of phosphoric acid from the phospho-peptide, so called neutral loss, and as a result poor sequence information, especially directly around the phosphorylated site. ETD does not induce dissociation of labile PTMs and the electron transfer results in fragmentation of the peptide backbone, giving superior phospho-site assignment (Swaney et al., 2009). However, only a subset of the phops data could be identified by both fragmentation methods, whereas the majority of the phops were identified by either ETD or CAD based fragmentation (Swaney et al., 2009; Grimsrud et al., 2010). To circumvent this problem, a decision tree-driven tandem MS algorithm was used to select the optimal fragmentation method for each precursor ion (Swaney et al., 2009; Grimsrud et al., 2010).

With the use of improved EDT methodology in plants and the identification of similar levels of pTyr in three plant model species Sugiyama's suggestion that tyrosine phosphorylation is as significant in plants as it is in animal systems seems to be confirmed. The significance of tyrosine phosphorylation for plant signaling has previously also been investigated by immunodetection using pTyr specific antibodies. Ghelis et al. (2008) used such an approach to study the regulation of Tyr phosphorylation in ABA transduction pathways. Proteins extracted from ABA treated Arabidopsis seeds were separated by two-dimensional gel electrophoresis and the changes in tyrosine phosphorylation levels were detected by using an anti-phosphotyrosine antibody on Western blot. A total number of 19 pTyr containing proteins were detected. The changes in pTyr levels of proteins obtained by immunoblot were confirmed by MALDI-TOF-TOF MS (Ghelis et al., 2008). In another study tyrosine phosphorylation of the BRI1 receptor kinase was characterized by using anti-phosphotyrosine antibodies. BRI1 was initially characterized as an autophosphorylating serine/threonine kinase, but recent work by Oh et al. (2009) has shown that BRI1 is also autophosphorylated on tyrosine residues. In our group we have conducted a targeted phosphoproteomics approach to specifically analyze pTyr signaling events. We used pTyr specific antibodies to immunoprecipitate Tyr phosphorylated peptides, implementing a methodology that has been previously described for animal systems (Blagoev et al., 2004; Lemeer et al., 2007). With our approach we were able to identify, with high confidence, 149 pTyr peptides (Mithoe et al., 2012). Our results are thus also in support of more significant levels of tyrosine phosphorylation in Arabidopsis cells, in contrast to the number of pTyr site identified in our initial study (Benschop et al., 2007).

Tyrosine phosphorylation in plants, is it more than a numbers game?

The question of how much pTyr may not be as relevant as the question what makes pTyr signaling so special in metazoans and could this also apply to plants. An interesting perspective on this subject was recently provided by looking at the evolution of pTyr signaling (Lim and Pawson, 2010). In metazoans pTYR signaling is regulated by a three-part tool kit; TSK, PTP and Scr Homology 2 (SH2) domains. Lim and Pawson (2010) describe this tool kit as the writer/reader/eraser module that can be used to build complex signaling circuits based on positive and negative feedback loops. This tool kit is only fully developed in metazoans and the unicellular Choanoflagellates that have about 40 PTP, about 100 SH2 and 50-100 TSK. Evolution of this toolkit is described to have occurred in three stages. In the first stage the early eukaryotic ancestor has only a few PTP and a prototypical SH2 domain that is not selective for pTyr. Stage 1 is currently exemplified by the situation in yeast. In stage 2 of the evolution the SH2 domain becomes selective for pTyr and is incorporated into simple multi-domain architecture proteins. This stage in the evolution is also accompanied by more DSK activity. This stage 2 version of the writer/reader/eraser system represents a very primitive form. The PTP family has not diversified yet and TSKs have not evolved, limiting the uses of this tool kit. This very primitive version of the writer/reader/eraser system is currently examplified by the situation in Slime mold *Dictostelium discoideum*. In stage 3 of the evolution, TSK domains evolve and expand and with concomitant expansion of both the PTP and the SH2 leads to a writer/reader/eraser system that allows a novel way of signaling based on tyrosine phosphorylation. It is only when TSK domains evolve that the full functionality of the PTP and SH2 domains can be exploited. The benefit of a pTyr specific signaling system that is based on three newly evolved domains is that it could have been implemented on top of existing signaling cascades without interfering with existing signaling networks and would have allowed new and complex signaling input to be transduced. Stage three is exemplified by the current state in metazoans and Choanoflagellates (Lim and Pawson, 2010).

When we survey the available plant genomics data it is immediately apparent that plants have not evolved along the three stage of the writer/reader/eraser system. Plants have only a few PTP and four proteins with predicted SH2 domains (Williams and Zvelebil, 2004; de la Fuente van Bentem and Hirt, 2009) and few TSK (Miranda-Saavedra and Barton, 2007). In this respect plants resemble yeast cells, although the latter do not have TSKs, but these may have been lost in the fungal lineage since diverging from the last common eukaryotic ancestor. However since the relative amount of tyrosine phosphorylation is not significantly different from animal systems, plants may have evolved a different strategy to read and write pTyr modifications. In this repect it is interesting that an additional pTyr binding domain has recently been identified in protein kinase C delta from mammalian cells (Miranda-Saavedra and Barton, 2007). Domains similar to C2 domains have also been identified in a substantial number of plant proteins, but the functionality as pTyr binding has yet to be tested (Miranda-Saavedra and Barton, 2007). The observation that C2 domains

may be present in plants does leave room for an altogether different system to read and write tyrosine phosphorylation in plants. In such a system the reading could be mediated by C2 domains, the writing by DSK domains and the erasing could be done by PTP domains. To constitute a functional alternative reader/writer/eraser system for pTyr signaling in plants, C2 domains would be expected to be in proteins with either phosphatase domains or kinase domains. However, C2 domains have not been identified in association with either domain in the same protein. Yet another interpretation could be that instead of developing a specific tyrosine phophorylation tool kit to add additional complexity to signaling networks, plant may have chosen to diversify their protein kinase tool kit, resulting also in increase numbers of DSKs and thus more tyrosine phosphorylation. When we take Arabidopsis for example the repertoire of RLK is about 630 and total number of annotated kinases well over a thousand, consistent with diversification of the kinase family as compared to metazoans. This hypothesis implies that the use tyrosine phosphorylation in plants has not evolved to fulfill a special regulatory role. The future challenge will be to obtain sufficiently large phosphoproteomic data sets, couple them to protein-protein interaction network analyses as well as computational motif analysis. This should point out in the near future whether a plant specific pTyr reader domain exist or whether plants made due with more kinase substrate connections to deal with complex signaling input.

CONCLUSION

The rapid development of the plant phosphoproteomic field is evident from the growing number of publications describing increasingly large-scale phops data sets. Almost all of these publications concern so called shotgun proteomic approaches aimed to discover as many new phosphorylation sites from plant cells from a specific tissue or under a specific condition. In addition to phosphorylation events that may be specific for the tissue or change under the selected condition, the fast majority of the identified phops will not necessarily be related to the question under investigation. Currently, the major challenge for the plant phospho-proteomic field is how to identify the relevant phosphorylation sites from the vast majority of phops. Implementation of quantitative approaches is an important development that will help to identify these relevant phosphorylation sites in proteins of interest to the investigator. A number of limitations of quantitative approaches are known to affect their success rate. First and foremost the ability to consistently observe and identify individual phops during replicate analysis. Since shot gun proteomic approaches use data dependent analysis to select precursor ions for tandem MS analysis the complexity of the mixture of peptide ions in the survey scan and as well as their relative abundance will determine whether a particular peptide ion is selected and identified. Variations in enrichment and fractionation between samples as well as between replicate analyses of the same sample affect this process. The inability to observe and identify a phop during tandem MS analysis is therefore not conclusive evidence of the absence of a phop from a particular sample.

This has a significant impact on most commonly used relative quantification approaches as each phop has to be observed and quantified in both samples that are compared (Fig.1). Similar limitations also apply to temporal analysis and are compounded when quantitative temporal analyses are undertaken. Improved enrichment and fractionation protocols have helped reduced complexity of the samples and enhanced the comprehensiveness of the phosphoproteome analysis. However no single enrichment strategy is capable of completely capturing the full complement of phops in any particular sample. Therefore, further development of improved enrichment strategies and development of novel enrichment strategies is still needed. Development of strategies using phospho-amino acid specific antibodies to specifically isolate phops is one potential novel approach that has been implemented for pTyr containing peptides. Currently this is not yet possible pSer or pThr due to the lack of suitable antibodies. Further development and implementation of fragmentation methods is another area that can help increase the comprehensiveness of phosphoproteome analysis, as recently shown (Swaney et al., 2009; Grimsrud et al., 2010). Fragmentation based on CAD is now complemented with newly developed ETD methodology and each method seems suitable for a specific subset of phops, but only some phops can be identified by both CAD and ETD. Parallel tandem MS analysis using both CAD and ETD based fragmentation will provide the most comprehensive phops identification and in cases where MS instruments can do both a decision tree-driven tandem MS algorithm as implemented by Swaney and colleagues (2009) seems very promising.

On the biological side the methodology to test the relevance of changes in phosphorylation is limiting. Functional analysis based on mutagenesis of phosphorylated residues in proteins is time consuming and is dependent on the availability of knock-out mutant lines to complement with loss- and gain-of-function substitution versions of phosphorylated proteins. These analyses can only be done on a case by case basis. However, it is possible to piece together a complete pathway as was shown for the brassinosteriod signal transduction pathway.

This effort will be aided by novel proteomics efforts, including selective reaction monitoring (SRM) which is a targeted MS approach (Domon and Aebersold, 2010). SRM is a relatively new targeted MS approach that selectively monitors and analysis predefined analytes (peptide ions) in complex mixtures. This makes it possible to focus only on changes in specific (phospho)proteins and ignore all the irrelevant peptide ions. SRM requires detailed pre-existing knowledge on all the reactions that are monitored, including chromatographic retention time of the peptide, m/z ratio of precursor ion and fragmention induced product-ions for the specific instrumentation used (Domon and Aebersold, 2010). When al this data is acquired it is possible to use SRM to monitor al (phospho)proteins that may together constitute a pathway, as was recently done for all yeast kinases and phosphoatases (Picotti et al., 2010). This targeted proteomics approach promises to be the next big thing for the (plant) phosphoproteomic field and in time may help to significantly advance plant signal transduction research beyond its current limitations.

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CHAPTER 3

3

Targeted quantitative phosphoproteomics approach for the detection of phospho-tyrosine signaling in plants

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ABSTRACT

Tyrosine (Tyr) phosphorylation plays an essential role in signaling in animal systems. However, a few studies have also reported Tyr phosphorylation in plants, but the relative contribution of tyrosine phosphorylation to plant signal transduction has remained an open question. We present an approach to selectively measure and quantify Tyr phosphorylation in plant cells, which can also be applied to whole plants. We combined a ^{15}N stable isotope metabolic labeling strategy with an immuno-affinity purification using phospho-tyrosine (pY) specific antibodies. This single enrichment strategy was sufficient to reproducibly identify and quantify pY containing peptides from total plant cell extract in a single LC-MS/MS run. We succeeded in identifying 149 unique pY peptides originating from 135 proteins, including a large set of different protein kinases and several receptor-like kinases. We used flagellin perception by Arabidopsis cells, a model system for pathogen triggered immune (PTI) signaling, to test our approach. We reproducibly quantified 23 pY peptides in two inversely labeled biological replicates identifying 11 differentially phosphorylated proteins. These include a set of 3 well-characterized flagellin responsive MAP kinases and 4 novel MAP kinases. With this targeted approach we elucidate a new level of complexity in flagellin-induced MAP kinase activation.

Keywords

Tyrosine, phosphoprotein, proteomics, Arabidopsis, metabolic labeling, quantification, mitogen activated protein kinase, flagellin, immune signaling

INTRODUCTION

Reversible protein phosphorylation is a key regulatory post-translational modification involved in cellular signaling processes in eukaryotic cells (Kersten et al., 2009). Changes in phosphorylation status can lead to conformational changes in the protein, affect protein-protein interactions and cause changes in protein activity (Pawson, 2004). Modulation of the protein activity may also lead to changes in the subcellular localization (Lee et al., 2004; Schulze, 2010). An example of phosphorylation induced change in the conformational state of the protein and the resulting change in activity, is the phosphorylation of the activation loop of protein kinases (Seet et al., 2006).

The identification of phosphorylated residues of proteins is the first step in gaining insight in cellular regulation. In the past decade several enrichment methods for efficient measurement of phosphorylation sites of phosphorylated proteins have been developed to measure the post translational modifications from complex protein mixtures (Ficarro et al., 2002; Blagoev et al., 2003; Nuhse et al., 2003; Pinkse et al., 2004; Thingholm et al., 2009). This has been aided by enhanced sensitivity of mass spectrometers, which has made it possible to analyze *in vivo* protein phosphorylation at a proteome scale in diverse organisms, including plants. Furthermore, the recent development of quantitative proteomics approaches (Heck and Krijgsveld, 2004; Bantscheff et al., 2007), based on metabolic labeling, post-extraction labeling or label free quantification, has made direct comparison between different signaling states possible (Schulze and Usadel, 2010). Such relative quantification approaches to study different conditions have also been used in plants (Schulze, 2010). Two different elicitors were used in a large-scale quantitative phosphoproteomic approach to directly compare site-specific modifications of proteins in plants before and after treatment (Benschop et al., 2007). This study and other early phosphoproteomic analyses in Arabidopsis mainly identified phosphorylation on serine and threonine residues (Nuhse et al., 2003; Benschop et al., 2007; de la Fuente van Bentem et al., 2008). The classic view is that phosphorylation events in eukaryotic cells, including plants, predominantly occurs on Serine (Ser), Threonine (Thr) and much less so on Tyrosine (Tyr) residues (de la Fuente van Bentem and Hirt, 2009; Mithoe and Menke, 2011). Despite the recent rapid developments in the field of plant signal transduction and the increase in sensitivity of mass spectrometers, it has been technically challenging to identify tyrosine phosphorylated residues by mass spectrometry (de la Fuente van Bentem and Hirt, 2009; Mithoe and Menke, 2011). Together with the fact that plant genomes do not encode for typical transmembrane receptor-like tyrosine kinases similar to animals (de la Fuente van Bentem and Hirt, 2009; Kersten et al., 2009), this has lead to the assumption that the occurrence of tyrosine phosphorylation in plants is less frequent when compared to animals (Luan, 2002).

Even in animal systems tyrosine phosphorylation is significantly less frequent than Ser and Thr phosphorylations, contributing in between 0.5 and 3% of all detected phosphosites in recent large-scale shot-gun proteomics experiments (Van Hoof et al., 2009; Rigbolt et al., 2011). Nevertheless Tyr phosphorylation plays a significant role in metazoan

signaling cascades (Blagoev et al., 2004; Del Rosario and White, 2010; Lim and Pawson, 2010; Ding et al., 2011). The significance of Tyr phosphorylation in plant signal transduction is less well understood, although recently large-scale phosphoproteomic screens in plants, have suggested a similar level of occurrence of Tyr phosphorylation (Sugiyama et al., 2008; Grimsrud et al., 2010; Nakagami et al., 2010). Sugiyama et al. (2008) combined two different phosphopeptide enrichment methods and coupled them with high accuracy mass spectrometry to show a significant proportion of tyrosine phosphorylation in Arabidopsis cells, which was initially reported to be 4.3%. This number was adjusted in a follow up large phosphoproteomic screen from the same group to 2.9 % for Arabidopsis as well as showing a similar level of tyrosine phosphorylation in the monocot *Oryza sativa* (Nakagami et al., 2010). Grimsrud et. al. (2010) reported that 1.3% of the over 2500 identified non-redundant phosphorylation sites detected in *Medicago truncatula* roots were Tyr phosphorylated. These reports indicate that Tyr phosphorylation may be present in several plant species to a similar level as in mammals, but do not indicate whether tyrosine phosphorylation has a distinctive significance in plant signaling as compared to serine and threonine phosphorylation. In individual cases, tyrosine phosphorylation has been linked to specific signaling events in plants. Tyrosine phosphorylation of the BRI 1 kinase inhibitor occurs as a response to brassinosteroid perception. This leads to translocation of BKI1 from the membrane into the cytosol where an active signaling complex is formed (Jaillais et al., 2011). Tyrosine phosphorylation of mitogen activated protein kinases (MAPKs) MPK4 and MPK6 has been linked to their activation in response to perception of biotic or abiotic stress signals (Ichimura et al., 2000; Nuhse et al., 2000).

In large-scale quantitative shotgun proteomics experiments the relative low abundance of tyrosine-phosphorylated residues is a limiting factor to identify tyrosyl-phosphorylated peptides. A comprehensive study to gain insight into the overall dynamics of tyrosine phosphorylation after stimulation with the EGF growth factor was done in mammalian HeLa cells. In this study multiple tyrosine phosphorylated residues of the EGF receptor were identified (Blagoev et al., 2004). More recently a targeted approach based on immunoprecipitation (IP) of tyrosine phosphorylated peptides with phospho-tyrosine (pY) specific antibodies was introduced for the use in animal cells (Zhang et al., 2005; Rikova et al., 2007; Boersema et al., 2010). To investigate differential Tyr phosphorylation Boersema et al. (2010) combined the cost-effective post-extraction dimethyl labeling (Boersema et al., 2009b) and pY IP enrichment to identify over 1100 unique pY peptides from EGF stimulated HeLa cells revealing 73 EGF-regulated phoshotyrosine peptides (Boersema et al., 2010). The use of pY antibodies has also been shown to detect tyrosine phosphorylation in plant protein samples (Oh et al., 2009b). Based on these observations we adopted this approach to analyze the contribution of tyrosine phosphorylation in plant signaling in response to external stimuli. We combined ^{15}N stable isotope metabolic labeling of plant cells with pY IP enrichment of tyrosine-phosphorylated peptides. We were interested in gaining insight in the innate immunity cascade, and therefore used a general elicitor of plant defense responses flagellin22 (flg22) to test our approach. Flg22 is a typical Microbe Associated

Molecular Pattern (MAMP) which is recognized through the Pattern Recognition Receptor (PRR) kinase FLS2 (Chinchilla et al., 2007). Upon recognition, several defense responses, which are important for the host immunity, are activated (He et al., 2007; Shan et al., 2007; Cui et al., 2009; Segonzac and Zipfel). This requires a signaling cascade that includes MAPKs. MAPKs are involved in many signaling cascades in plants, including the protection against microbial invasion (Colcombet and Hirt, 2008; Pitzschke et al., 2009; Rodriguez et al., 2010). Flg22 perception leads to phosphorylation of MAPKs and these modifications are required for their activation (Nuhse et al., 2000; Asai et al., 2002; Menke et al., 2004).

This is to our knowledge the first time that a combination of metabolic labeling strategy of plant cells is followed by a phosphotyrosine IP. We identified from a complex protein mixture the well-known key players of plant defense, such as MPK6, MPK4 and MPK3, as differentially phosphorylated on tyrosine residues. We also identified several other protein kinases involved in defense related responses.

RESULTS

Enrichment of tyrosine phosphorylated peptides

Successful and reproducible measurement of tyrosine phosphorylated residues in peptide fractions obtained from plants is essential for quantitative analysis of tyrosine phosphorylation. Shotgun proteomics approaches selects only the most abundant peaks from the full spectrum survey scan for fragmentation and MS/MS analyses. The low abundance of tyrosine phosphorylated residues in plant cell extracts limits the chance of identification of these residues from complex phospho-peptide enriched fractions. Therefore we set up an enrichment strategy that would specifically be targeted towards enrichment the pY containing peptide fraction and allowed us to measure peptides with pY more reproducibly. We first tested our protocol on suspension cultured Arabidopsis cells treated with flg22 and mock treated cultures growing on normal culture media. Cell cultures were treated and total protein extracted as described before (Benschop et al., 2007). Total protein extracts were digested with trypsin and the tryptic digests were enriched with an anti-phospho-tyrosine antibody immobilized on agarose beads. The peptide fractions were eluted from the agarose resin followed by LC-MS analysis on a LTQ-Orbitrap. Two independent biological replicate experiments were done, as described in more detail in the Methods section. For each biological replicate, several independent IPs were done on mock and flg22-treated cell extracts and analyzed by LC -MS/MS. As noted before, immunoprecipitated peptide fractions included pY containing peptides (data available in PRIDE database under accession numbers 19496, 19497, 19498). In total 11 LC-MS/MS runs were done and we identified 149 unique pY containing phosphopeptides from these combined LC-MS runs (Supplemental Table 1, Supporting information). These phospho-peptides map to 135 proteins and a

substantial number (40%) of the identified pY peptides were also phosphorylated on Ser or Thr residues, similar to what has been previously reported (Sugiyama et al., 2008).

Comparison against databases

To explore the overlap with previously reported pY datasets retrieved in high throughput phophoproteomics screens, we made a comparison against the Arabidopsis phosphoproteome database PhosPhAt 3.0 (Heazlewood et al., 2008; Durek et al., 2010). This database contains the most comprehensive collection of phosphorylated peptides identified by mass spectrometry from Arabidopsis. These comparisons revealed that 8 pY containing peptides were identified in previous studies (Table 1).

Table 1: Previously reported pY phosphorylated peptides in phosphotyrosine proteome dataset.

Gene identifier	Peptide	Gene description
AT1G09840.1	GEPNVSpYICSR	SHAGGY-LIKE PROTEIN KINASE 41 (SK41)
AT1G71410.1	pYMLFVKDILR	ARM repeat superfamily protein
AT2G40120.1	SDNLCLpYVQSR	Protein kinase superfamily protein
AT2G43790.1	VTSESDFMTEpYVVTR	MAP KINASE 6 (MPK6)
AT3G09200.1	VEEKEESDEEDpYGGDFGLFDEE	Ribosomal protein L10 family protein
AT4G00720.1	MLVPGEPNISpYICSR	Shaggy-related protein kinase theta / ASK-theta (ASK8)
AT4G18710.1	QLVKGEANISpYICSR	BIN2 (BRASSINOSTEROID-INSENSITIVE 2); kinase
AT5G05440.1	VpYKNFIR	PYRABACTIN RESISTANCE 1-LIKE 5 (PYL5)
AT5G35980.1	TVYSpYIQSR	YEAST YAK1-RELATED GENE 1 (YAK1)
AT5G37970.1	pYVNYFIVLKR	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein

In addition, we identified 2 peptides (MLVPGEPNISpYICSR and QLVKGEANISpYICSR) containing phosphorylated tyrosine residues that were previously identified in similar, but not identical phosphopeptides (as a result of miscleavages). Therefore the vast majority (139) of the pY phosphopeptide identified in our study are novel pY containing phosphopeptides. The limited overlap of our dataset with the PhosPhAt database could be due to the incomprehensive nature of the phosphotyrosine proteome in the database, as well as due to the incomprehensive nature of our dataset. In addition to this, some of the pY residues identified in our set could be specific for the conditions of our system, in which cell cultures are treated with flg22. Furthermore, it is likely that the different enrichment procedures to enrich phosphopeptides, eg. metal oxide affinity chromatography (MOAC) versus IP using pY specific Ab, contribute to overlapping but distinct subsets of the pY phosphoproteome (Boekhorst et al., 2011).

Classification of tyrosine phosphorylated proteins

We analyzed our pY containing phosphoprotein dataset for localization, molecular function and biological processes at a stringent Mascot score threshold of 35 (Fig. 1) and more relaxed cut-off of 20 (Supplemental Figure 1, Supporting information). Analyses using Arabidopsis Gene Ontology (GO) slim annotations revealed that the largest group of the identified pY proteins, 30.4% is annotated to be localized in the cytoplasm and cytosol (Fig. 1A). These categories were also shown to be significantly overrepresented by a hyper geometric test of overrepresented GO Slim Plants ontology terms with a Benjamini & Hochberg False Discovery Rate (FDR) correction (Table 2). In addition, we classified and categorized the representation of the molecular function of the pY containing peptides in our dataset as compared to the annotated Arabidopsis proteome. This revealed that 41.5% of the identified pY proteins are annotated to have kinase activity as compared to 4.6% in the predicted Arabidopsis proteome. (Fig. 1B). This was also confirmed by a hypergeometric test of overrepresented GO Slim Plants ontology terms with FDR correction (Table 2). Other terms related or associated with kinase activity, such as transferase activity and signal transducer activity were also overrepresented (Table 2). This also correlates well with the overrepresentation of protein modification process and signal transduction (Table 2).

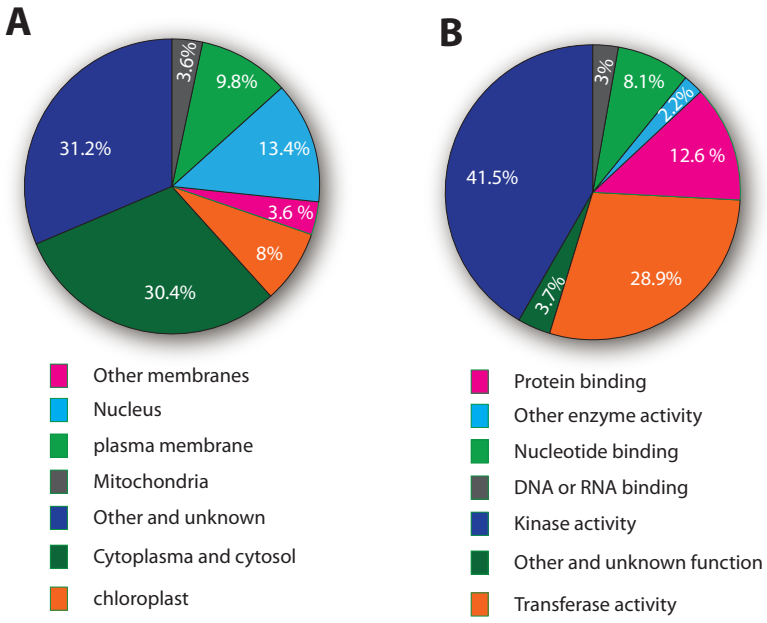

Figure 1. Representation of GO slim annotations for the identified Tyr phosphorylated proteins. **A)** Pie chart showing the distribution of annotated cellular localizations. **B)** Pie chart showing the distribution of annotated molecular functions.

Table 2: **GO annotations with significant overrepresentation in phosphotyrosine proteome dataset.**

GO category	GO annotation	Corrected *p*-value
Molecular function	Kinase activity	6.72E-27
Molecular function	Transferase activity	3.04E-19
Molecular function	Catalytic activity	1.41E-10
Molecular function	Signal transducer activity	6.61E-15
Molecular function	Nucleotide binding	3.55E-05
Molecular function	Binding	1.56E-02
Biological process	Signal transduction	1.75E-13
Biological process	Protein modification process	1.65E-06
Biological process	Cellular process	4.86E-05
Biological process	Metabolic process	7.28E-04
Biological process	Protein metabolic process	3.17E-04
Biological process	Response to endogenous stimulus	5.55E-07
Biological process	Response to abiotic stimulus	5.53E-05
Biological process	Response to stress	5.53E-05
Cellular compartment	Cytosol	1.51E-06
Cellular compartment	Mitochondrion	4.95E-02
Cellular compartment	Cytoskeleton	4.95E-02
Cellular compartment	Plasma membrane	2.55E-03
Cellular compartment	Nucleus	6.52E-03

The outcome of this analysis did not significantly change when we used the larger data set with a more relaxed Mascot score cut-off at 20 (Supplemental Figure 1 and Supplemental Table 3, Supporting information). These results indicate that proteins with signal transduction activity and/or protein kinase activity are a major class of targets of tyrosine or dual specificity kinases. A similar overrepresentation for protein kinases was also observed in another phosphoproteomic Arabidopsis dataset describing a significant amount of pY peptides (Sugiyama et al., 2008). However in Sugiyama et al. (2008) the significance of overrepresentation was not tested. These observations suggest that the respective protein kinases are regulated by phosphorylation on tyrosine residues. One such class of kinases known to be regulated via tyrosine phosphorylation is the MAP kinases. In our data set MAP kinases are prominently represented, together with the related Glycogen synthase kinase (GSK) class. This is observed both at the level of the number of MAPK and GSK proteins as well as the number of times the respective pY containing peptides derived from MAPK or GSK are observed. The number of spectra observed for pY peptides of MAPK's or GSK's in the combined dataset represents a very significant amount of all spectra measured in our study.

Evolutionary conservation of Tyr phosphorylation

To address whether tyrosine phosphorylated residues are conserved we compared conservation of the tyrosine residue and surrounding amino acids to Arabidopsis paralogues and orthologues in other dicot species (Fig. 2). The complete lists of the peptide sequences with Mascot score >= 35 (Fig. 2) or Mascot score >= 20 (Supplemental Figure 2, Supporting information) were compared against Phytozome database v7.0 which included sequences of the following genomes: *Arabidopsis thaliana, Arabidopsis lyrata, Carica papaya, Medicago truncatula, Populus trichocarpa, Glycine max, Vitis vinifera, Mimulus guttatus, Ricinus communis, Cucumis sativus, Prunus persica, Eucalyptus grandis, Manihot esculenta* and *Citrus clementina.*

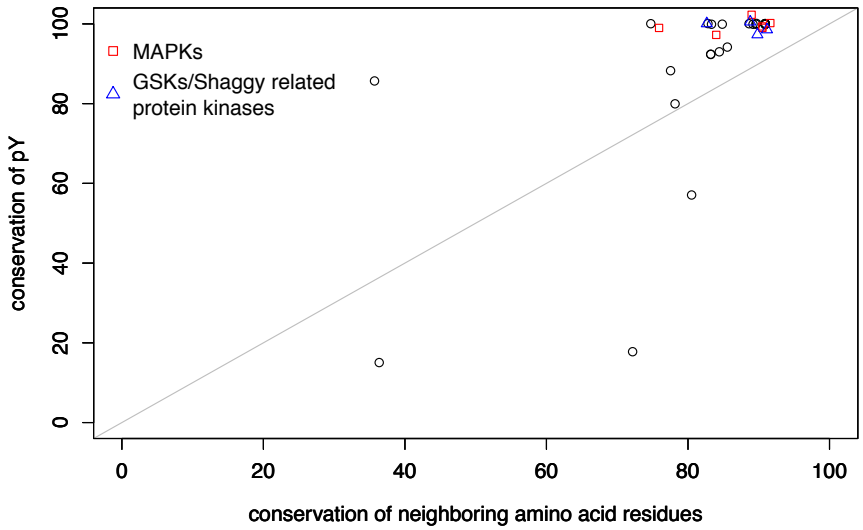

Figure 2. Evolutionary conservation of phosphorylated Tyrosine residues. The conservation of phosphorylated tyrosine and surrounding amino acids was determined by comparison of corresponding phospho-proteins to orthologues in species in the Phytozome database. The percentage conservation of the phosphorylated tyrosine residue (y-axis) is plotted against the conservation of 5 surrounding residues on each side of the tyrosine residue (x-axis). The diagonal in the graph represents equal conservation of tyrosine residue and surrounding residues over the entire range of conservation levels. MAP kinases are represented by red squares; GSKs are represented by blue triangles.

The largest proportion of the phosphorylated tyrosine residues in the proteins we measured in Arabidopsis was highly conserved in the orthologous species, with 33 of 36 peptides showing > 80% conservation of the Tyr residue (Fig. 2). In this category we distinguished conserved Tyr residues in peptides that also showed high levels of conservation of the surrounding residues (upper right-hand corner) as well as 1 peptide with conserved Tyr

residues surrounded by AA that showed less then 40% conservation. When peptides identified based on a Mascot score between 20 and 35 were included, the trend remained the same, with 100 of 149 peptides showing > 75% conservation of the Tyr residue (Supplemental Fig. 2, Supporting information). In this category we distinguished conserved Tyr residues in peptides that also showed high levels of conservation of the surrounding residues (indicated by yellow ellipse) as well as 10-15 peptides with conserved Tyr residues surrounded by AA that show only 40-50% conservation (indicated with blue ellipse). This demonstrates that tyrosine phosphorylated residues are highly evolutionary conserved, even in regions showing relatively lower overall conservation. This observation also contributes to the idea that the amino acids surrounding the pY site could contribute to the recognition of the conserved sites of protein kinases (Nakagami et al., 2010). In itself, this could provide a positive selective force for the evolutionary conservation of the surrounding amino acids. The overall high level of conservation of AA sequences surrounding the pY residue is different from the conservation observed for phosphorylated Ser residues, which were previously shown to be less conserved in plants (Nuhse et al., 2004). A significant number of the proteins with high conservation of both the tyrosine residues and the surrounding residues are protein kinases of the MAPK and GSK family. These observations are in line with the notion that the pY sites of these protein kinases are important for the regulation of their activity and that the surrounding amino acids are contributing to the recognition sequence for the upstream protein kinases and are structurally important for the phosphorylation dependent modulation of the kinase activity.

In addition to these two families of protein kinases with highly conserved pY residues, we also identified 5 protein kinases that are classified as Receptor Like Kinases (RLKs). Plant genome annotations have indicated that plants have large families of RLKs, however these have independently evolved from receptor like kinases in metazoan species. In metazoan species most receptor kinases have been annotated as Tyrosine Receptor Kinases (TRKs) where as plant RLKs are consistently annotated as Serine/Threonine kinases (Gish and Clark, 2011). Recent individual analyses of a few RLKs involved in brassinolide perception using pY antibodies and mutagenesis of tyrosine-phosphorylated residues have shown phosphorylation on tyrosine residues. (Oh et al., 2009a; Oh et al., 2009b; Oh et al.). The phosphorylated tyrosine residues in these RLKs required endogenous kinase activity, indicating that the tyrosine phosphorylation is the result of trans-phosphorylation. The 5 new RLKs with phosphorylated tyrosine residues we identify here (Supplemental Table 4 and Supplemental Table 1, Supporting information) were identified based on peptides that did not qualify for the data set with a stringent Mascot score cut-off of >=35. Further experimental evidence will be required to validate these as tyrosine phosphorylated RLKs. Nevertheless, the possible identification of these 5 RLKs as tyrosine phosphorylated proteins could also lend support to the notion that more plant RLKs than previously recognized, function as tyrosine kinase or dual specificity kinases.

Quantitative analysis of tyrosine phosphorylation

The above general analysis of our dataset suggests that the set of tyrosine phosphorylated proteins we identified has similar characteristics as a previously identified and partially overlapping pY data set (Sugiyama et al., 2008; Nakagami et al., 2010). We next combined the pY IP enrichment method with metabolic labeling of the Arabidopsis cells to implement a novel quantitative pY enrichment approach for plant signaling (Fig. 3).

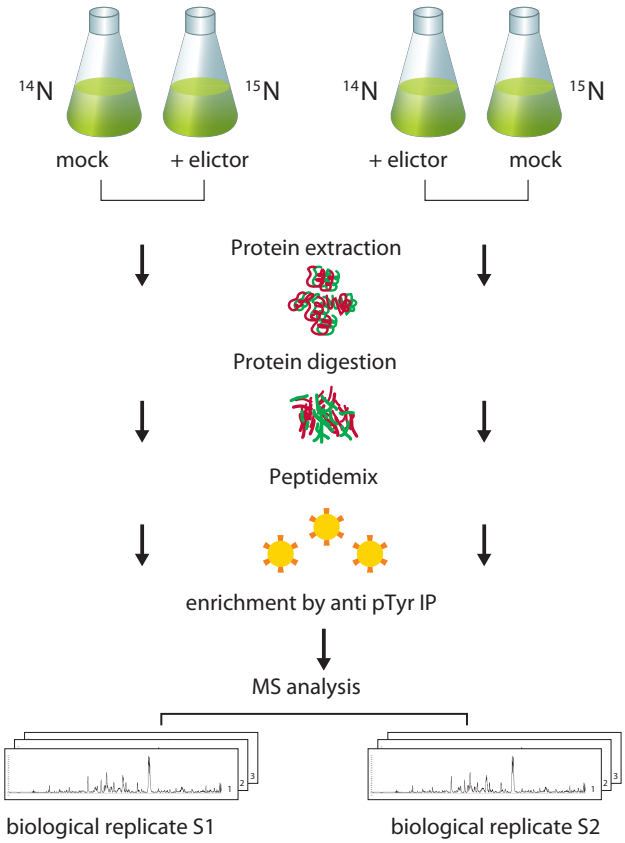

Figure 3. Workflow of the targeted quantitative phosphotyrosine proteomics approach. Suspension cultures were grown on basal salt media containing ^{15}N stable isotopes or natural occurring ^{14}N. Cultures were treated as indicated and pooled at a 1 to 1 ratio. Proteins were extracted from pooled samples, and cleared extracts were digested with trypsin. Peptide lysates were incubated with immobilized pY-specific antibodies and enriched by immunoprecipitation. The precipitated peptides were analyzed by LC-MS/MS.

The metabolic labeling strategy allowed us to directly compare changes in phosphorylation status of proteins of mock-treated and elicitor-treated cells (Benschop et al., 2007). Four subculture periods of 7 days were sufficient for near-complete incorporation (>99%) of ^{15}N

in to randomly selected proteins (Supplemental Fig. 3, Supporting information). The isotope incorporation rates were monitored by measurement on LC-MS. Spectra obtained from tryptic digests were compared with theoretical spectra representing 99.5% incorporation rates. Since the observed and theoretical spectra were nearly identical we concluded that we achieved > 99% incorporation rate of ^{15}N in the proteins of these Arabidopsis cells.

As a test case for our approach and to gain more insight in the phosphotyrosine phosphorylation dynamics involved in PTI signaling, we induced the cell cultures with flg22. This general elicitor of plant defense-associated responses is used as a model system to study primary defense signal transduction pathways. Our previous study (Benschop et al., 2007) demonstrated that a short time frame of 10 min of elicitor induction is sufficient to detect early signaling events, without changes of the protein abundance. As before, we treated the cell cultures with 1 µM flg22 for 10 minutes. The elicitor treated cells grown on ^{14}N medium were pooled and mixed with an equal amount of ^{15}N mock-treated cells. The pooled cells were homogenized in ice-cold extraction buffer. Additionally we performed an inverse labeling experiment with elicitor induction of the ^{15}N grown cells and mock-treatment of the ^{14}N grown cells. We mixed equal amounts of the labeled cells and this made our measurements independent of the variation introduced by metabolic labeling and insured a biologically relevant high confidence data set.

Total protein extracts were digested with trypsin as described previously and two independent IPs with anti-pY antibodies were done for each tryptic digests. This resulted in 2 technical replicates for each of the 2 biological replicates and these fractions were analyzed separately by LC-MS/MS. The 4 LC-MS fractions were analyzed by the Mascot search engine. Peptides were included in the data set when they had a Mascot threshold score of 20 in order to reduce the probability of inclusion of false positives. The relative quantification ratio's of the identified phosphopeptides, was determined by extraction of the ion chromatograms of the ^{14}N and ^{15}N forms of the peptide. We identified and quantified a total set of 23 tyrosine phosphorylated peptides mapping to 22 tyrosine phosphorylated proteins (Table 3 and Supplemental Table 1, Supporting information). The number of quantified peptides did not allow significance testing to determine a cutoff for differential phosphorylation. Thus we defined differential phosphorylation by applying a 2-fold cutoff.

Differentially phosphorylated proteins

Interestingly we identified a large set of MAPKs, which are known as Ser/Thr kinases. With our targeted approach we detected 10 out of 20 known MAPKs phosphorylated on the Tyr residue of their T-x-Y motif. Sequence comparison of the conserved TxY, which is phosphorylated by MKKs shows two subtypes. The TEY subtype of the MAPks is based on their phylogenetic relationship classified into group A,B and C (Group et al., 2002; Jonak et al., 2002). The more distant TDY amino acid subtype is classified as group D MAPKs. Group A MAPKs MPK3 and 6 are known as positive regulators of plant defense signaling (Asai et al., 2002; Menke et al., 2004). These two MAPKs and another set of 2 group B MAPKs, namely

Table 3: Quantified tyrosine phosphorylated peptides from flg22 treated cells.

Peptide sequence	Gene identifier	Protein name	Average ratio flg22/mock (log2)	Stdv
ICDFGLARPTSENDFMTEpYVVTR	AT3G45640.1	ATMPK3	5.96	0.96
TSNETEIMTEpYVVTR	AT1G07880.1	ATMPK13	4.89	0.97
VTSESDFMpTEpYVVTR	AT2G43790.1	ATMPK6	3.94	2.3
VTSESDFMTEpYVVTR	AT2G43790.1	ATMPK6	3.92	0.65
SETDFMTEpYVVTR	AT1G01560.1	ATMPK11	3.71	
VSFNDAPTAIFWTDpYVATR	AT1G18150.1	ATMPK8	1.58	0.26
VSFNDAPSAIFWTDpYVATR	AT3G18040.1	MPK9	1.26	0.22
ASEDSDSpYDDYVK	AT3G52870.1	Calmodulin-binding	1.16	0.58
VSFTDSPSAVFWTDpYVATR	AT2G01450.1	ATMPK17	0.99	0.28
SGGGGGYSGGGGGGpYSGGGGGGYER	AT4G39260.1	ATGRP8/GR-RBP8	0.88	0.17
GQFMTEpYVVTR	AT1G10210.1	ATMPK1	0.26	0.22
TVYSpYIQSR	AT5G35980.1	Protein kinase family protein	0.06	0.15
NEVTPpYLVSR	AT3G25840.1	Protein kinase family protein	0.05	0.17
GEPNISpYICSR	AT1G06390.1	GSK1	0.02	0.06
ILFLDLTSDPpYLFKGPK	AT1G09450.1	Haspin-related	0.01	0.32
VAFNDTPTTIFWTDpYVATR	AT2G42880.1	ATMPK20	-0.01	0.56
GEANISpYICSR	AT2G30980.1	Shaggy-related delta	-0.02	0.15
GEPNVSpYICSR	AT1G09840.1	Shaggy-related kappa	-0.06	0.04
SDNLCLpYVQSR	AT2G40120.1	Protein kinase family protein	-0.11	0.09
ILFLDLTSDPpYLFK	AT1G09450.1	Haspin-related	-0.18	0.28
TPNIEPQGpYSEEEEEEEEEVPAGNAAK	AT3G44750.1	HD2A	-0.2	
VIDLGSSCFETDHLCSpYVQSR	AT1G73450.1	Protein kinase, putative	-0.39	0.89
VAFNDTPTAIFWTDpYVATR	AT5G19010.1	MPK16	-0.5	0.22

MPK4/11 and 13 are high differentially phosphorylated on the Tyr residue (Table 3). MPK4 has been linked to defense responses, but as negative regulators of defense response (Petersen et al., 2000). MPK4 has also been reported being activated in response to flg22 perception (Meszaros et al., 2006; Suarez-Rodriguez et al., 2007). Surprisingly only one group A MAP kinase, MPK6, was dually phosphorylated on both the tyrosine and the threonine residues in our quantitative dataset. We have also observed dual phosphorylation of MPK4/11, but this observation was not quantified (Supplemental Table I, Supporting information). All spectra for the pY peptides of the observed MAPKs in our data set were manually inspected and only MPK6 was verified to show both mono and dual phosphorylated TEY motive. Similar manual inspection was also done for two data sets derived from human HeLa cells stimulated with EGF and human Embryonic Stem Cells stimulated with FGF to assess the significance of these observations (Boersema et al., 2010; Ding et al., 2011). In both data sets the mono phosphorylated pY containing peptide was more abundant than the dually phosphorylated pTXpY containing peptides derived from MAP kinases (Boersema et al., 2010; Ding et al., 2011). Since these datasets were also obtained by pY specific IP with similar antibodies, the

observed abundance of the monophosphorylated pY containing peptide over the pTXpY containing peptide could indicate a bias for this method. However, in all three data sets pTXpY containing peptides could be readily measured and an excess of antibody was used. This indicates that the observed levels of mono and dual phosphorylated TXY containing peptides are biologically relevant observations and could be suggestive of active conversion of these peptides. In mammalian systems dual phosphorylation of both Thr and Tyr residue is required for maximum activation of the MAPKs. This mechanism for maximum activation is also likely to be conserved in plants and we observe dual phosphorylation for both MPK4 and MPK6 in response to flg22 perception. However, MPK3 is also highly activated at 10 min post induction with flg22 (Fig. 4), but does not appear to be dually phosphorylated on both threonine and tyrosine residues. It appears that in plants tyrosine phosphorylation of the TEY motif of MPK3, MPK4 and MPK6 is sufficient for significant activation. Alternatively, the observed singly Tyr phosphorylated peptides for MPK3, MPK4 and MPK6 could be intermediates in the activation or inactivation. In independent plant pY containing datasets (Sugiyama et al., 2008; Nakagami et al., 2010) only monophosphorylated pY containing peptides from MPK4/11 and MPK6 were observed.

In these studies phospho-peptides were enriched with MOAC. Since these cells were not stimulated with a MAMP or abiotic stress, the observed pY residue could be the result of basal MAP kinase cascade activity. From these observations the suggestion goes out that monophosphorylated TXpY forms of MAPK may represent a lower activity state MAPK and that pTXpY represents the highest activation status of MAP kinases. This is consistent with MPK6 being the most highly activated MAP kinase in response to stress responses such as flg22 perception and wounding (Fig 4, (Menke et al., 2004)).

However, both the threonine and tyrosine residues are required for phosphorylation by the corresponding upstream MEK. Irreversible dephosphorylation of the threonine residues by pathogen effectors with phosphothreonine lyase activity leads to the complete inactivation of MAPKs (Li et al., 2007; Zhang et al., 2007) . We verified the phosphorylation of the tyrosine residue of MPK3, MPK6 and MPK4/11 with immunoblotting using two antibodies that recognize phosphorylated Y residues (Fig. 4B). Both the pY specific antibody and the antibodies raised against phoshorylated ERK1/2 detected phosphorylated MPK3, MPK6 and MPK4/11 in our samples. The latter antibodies specifically recognized pY in the pTXpY context, but it is well known that these antibodies can recognize also monophosphorylated epitopes, phosphorylated only on the Y residues in ERK1/2 and we speculate it also does so for MPK3 and MPK6 as well as MPK4/11. In addition, we also verified the flg22-induced activation of MPK3, MPK6 and MPK4/11 by immunoprecipitation with MPK3, MPK4 and MPK6 specific antibodies (Menke et al., 2004) followed by an *in vitro* kinase activity assay of the immunoprecipitated kinases (Supplemental Fig. 4, Supporting information). This verified the activation of MPK3, MPK6 and MPK4/11 by flg22 at 5 and 10 minutes post induction. MPK4 and MPK11 are paralogues that can not be distinguished with the antibody raised against the C-terminus of MPK4 (Menke et al., 2004). The close homology of these

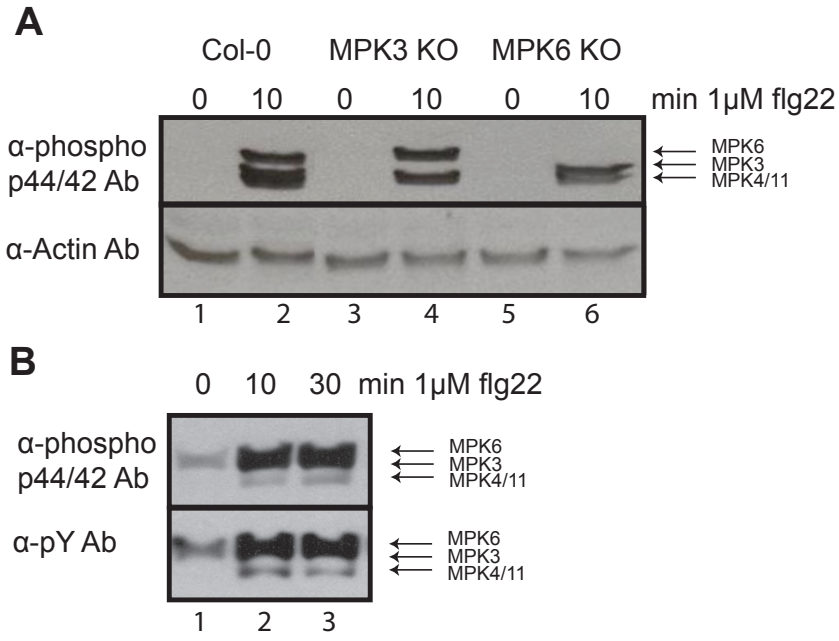

Figure 4. **Tyrosine phosphorylation of activated MAP kinase induced by flagellin. A)** Induction of Arabidopsis leaf strips with flagellin results in the phosphorylation of the T-x-Y motif of three MAP kinases. Upper panel shows immunoblot with an antibody raised against phospho Erk1/2 which detects three bands corresponding to MPK3, MPK4/11 and MPK6 in Col-0 wildtype at 10 min post induction (lane2). Similar induction in Knock Out (KO) mutant lines of MPK3 (lane 3 and 4) or MPK6 (land 5 and 6) show detection of 2 bands, corresponding to MPK6 and MPK4/11 (lane 4) or MPK3 and MPK4/11 (lane 6) respectively. Lower panel shows blot incubated with anti-Actin antibody as a loading control. **B)** Immunoblot with antibody raised against phospho Erk1/2 detects three bands corresponding to MPK3, MPK4/11 and MPK6 in cultured Arabidopsis cells, 10 min (lane2) and 30 min post induction (lane 3) with 1 mM flg22 (upper panel). Immunoblot with antibody raised against phospho-tyrosine detects three bands corresponding to MPK3, MPK4/11 and MPK6 in cultured Arabidopsis cells; 10 min post induction (lane2) and 30 min post induction (lane 3) with 1 mM flg22 (lower panel). Lane numbers are indicated below the lower panels in A and B.

paralogues also prevents the assignment of the identified phosphopeptide to one of the two paralogues.

A group of less studied group D MAPKs; MPK8, 9 and 17 were also differentially phosphorylated on the Tyr residue of the TDY motif. Up to date only MPK3, 4 and 6 are well studied and there is little information of the possible function of the group C and group D MAPKs. It may well be that the observed differential phosphorylation of the group D MAPKs is functionally relevant and that these MAPKs may play an as of yet unidentified positive or negative role in defense signaling. Recent evidence for the involvement of MPK8 in wound induced calcium signaling and negative regulation of ROS production has been reported (Takahashi et al., 2011). Based on this we speculate that flg22 dependent activation of

MPK8 may be required to attenuate ROS signaling, which was shown to be induced by flg22 perception (Schwessinger et al., 2011) We also identified pY phosphorylation of MPK1 and MPK20, but these MAPKs were not differentially phosphorylated. However, MPK1, MPK2, MPK7 and MPK14 belong to group C MAPKs and this group can be activated by MKK3 similar to MPK8 (Doczi et al., 2007; Takahashi et al., 2011). The module MKK3-MPK7 is linked to pathogen defense signaling (Doczi et al., 2007). However we do not detect MPK7 derived phospho-peptides and other group C MAPKs differentially phosphorylated in response to flg22 perception.

Our findings are consistent with the involvement of MAP kinases in FLS2 dependent signaling and validate our approach. The detection of differential and non-differential pY phosphorylated peptides of a large group of MAPKs points to a more complex response than hitherto appreciated and detected by conventional biochemical methods or large-scale shotgun phosphoproteomics approaches.

In addition to the large group of MAPKs, we also identified a calcium dependent, Calmodulin binding protein as differentially phosphorylated on the Tyr residue. Several shaggy related protein kinases and GSK1 are also phosphorylated on the pY residue, but they are not differentially phosphorylated. Some GSK family members are reported to be involved in Brassinosteroid signal transduction and our measurements indicate that these GSKs are not differentially phosphorylated on the tyrosine residue after inductions with a bacterial elicitor. BRI perception and flg22 perception both require BAK1 as a coreceptor but Tyr 610 phosphorylation is associated primarily with BR signaling and does not appear to be required for FLS2 mediated response (Oh et al., 2010b; Schwessinger et al., 2011). These data show that, although part of the signaling cascade is shared, downstream flg22-induced tyrosine phosphorylation events are specific for the FLS2 pathway.

We also measured tyrosine phosphorylation of CYCB1;4 (CYCLIN 3; AT1G34460) and CDKA1 (AT3G48750) a cyclin-dependent protein kinase involved in cell division control (Supplemental Table 1, Supporting information). This is consistent with the protein extracts deriving from actively dividing cells and suggests that tyrosine phosphorylation could also regulate aspects of the cell cycle. Applying our approach to synchronized cell cultures would allow the identification of tyrosine residues important for the regulation of the cell cycle.

DISCUSSION

Protein phosphorylation is considered to be a central mechanism for regulation and cellular signaling in eukaryotes. Tyrosine phosphorylation has been extensively studied in eukaryotes other than plants and has been described to have an important role in developmental processes and human diseases (Boersema et al., 2010; Del Rosario and White, 2010; Ding et al.). However, to our knowledge this is the first report in plants to undertake a quantitative phosphotyrosine screen to understand the tyrosine signaling events in plants. We combined a metabolic labeling method for plant cells with a phospho-tyrosine IP based enrichment

step. The objective of our study was to develop an approach for the targeted analysis of tyrosine phosphorylation in plants and to gain insight in the signaling processes focusing on the innate immunity pathway. By combining the IP based enrichment with metabolic labeling of plant cells, we were able to directly compare changes in phosphorylation in a complex cell mixture. With the recent improved development of effective ^{15}N metabolic labeling of entire seedlings (Bindschedler et al., 2008) this method can also be applied to pY signaling in intact plants.

Our method was cost-effective, since the labeling is done with relatively inexpensive ^{15}N labeled nitrate and ammonium salts as compared to more complex labeling protocols such iTRAQ or SILAC. Furthermore SILAC has additional disadvantages for the use in plants, as plants produce the amino acids used for labeling (Gruhler et al., 2005).

With our protocol we successfully measured phosphotyrosine phosphorylation of 135 proteins. Our dataset has a limited overlap with the phosphotyrosine phosphorylated proteins measured in earlier studies performed by Sugiyama et al. (2008) Nakagami et al. (2010), who reported 94 tyrosine phosphorylated proteins in Arabidopsis and those in the PhosPhAt data base, which contains 541 unique validated pY residues. In this study we report an additional 139 tyrosine phosphorylation sites, thereby increasing the total number of experimentally identified phosphorylated tyrosine residues in Arabidopsis with about 20%. Overall the total number of pY containing peptides we successfully identified in our study is similar to the number tyrosine phosphorylation reported by Nagakami et al (2010), which represents 2.9% of the total phosphoproteome. We were unable to compare our percentage of pY phosphorylation to percentages of pS and pT, as our method was specifically designed to enrich pY containing peptides.

CONCLUSION

We report a novel strategy, which can easily be applied to other stress conditions and responses to environmental changes to elucidate other signal transduction networks in plants. Our study provides a large quantitative pY Arabidopsis phosphoproteomics dataset and supports Sugiyama's original hypothesis that pY phosphorylation is much more abundant in plants. By just zooming in on pY phosphorylation in a complex cell mixture, the dataset reveals that the response to an elicitor by the plant is an orchestra of several MAPK pathways, indicating that a targeted phosphoproteomics approach is required to understand the full mechanism. We successfully combined two established methods to gain a first limited glimpse in pY phosphorylation events in relation to innate immunity pathways in plants. Temporal proteomics analysis based on our novel approach is a likely next step to gain insight in pY phosphorylation and dephosphorylation of the protein kinases involved in immune signaling in plants.

MATERIALS AND METHODS

Materials

$K^{15}NO_3$ and $(^{15}NH_4)_2SO_4$ were purchased from Spectra Stable Isotopes (Columbia, MD). Naphthaleneacetic acid (NAA) was purchased from Duchefa (The Netherlands). The peptide flagellin (flg22) was synthesized by Sigma Genosys.

Arabidopsis cell cultures and elicitor treatment

50 ml cell suspension cultures of *Arabidopsis thaliana* cells were grown in Gamborg B5 medium, supplemented with 30 g/liter sucrose and 1 µM NAA. Cells were grown in 250-ml flasks with shaking at 150 rpm, 8 h of light at 22 °C. Subculturing of the cells was done by transferring 6-8 ml culture into 50 ml new medium every 7 days. All experiments were performed at 4-5 days after subculturing. Cultures were grown for at least 4 subculture periods of 7 days on either ^{14}N- or ^{15}N-medium. The separately grown cultures were harvested and washed in ice-cold KCl buffer. For the elicitor treatment four-day-old cultures were treated for exactly 10 min with 1 µM flg22 (Sigma Genosys). For the first biological replicate, three separately flg22-treated (^{14}N) cultures and three mock-treated (^{15}N) cultures were pooled, harvested by filtration and washed with ice-cold 20 mM KCl buffer. For the second biological replicate the cell cultures were inversely labeled and treated as previously described.

Western blot and immunocomplex kinase assays

Western analysis was performed using duplicate blots in which equal amounts of 20 µg crude protein extracts from flg22-treated leaf strips were separated on 10% SDS-PAGE and transferred to nitrocellulose (Schleicher & Schuell) by wet transfer as described before (Menke et al., 2004). Antibodies were diluted in TTBS with 3% (w/v) bovine serum albumin (Sigma) to 1 µg/ml (α-C-4 and α-N-6) or 5 µg/ml (α-C-3). Western blots were incubated with diluted antibody for 16 hr at 4°C. Antibody pY99 (Santa Cruz Biotechnology Inc), antibody phospho-p44/42 (Cell signaling technology) and mouse α-actin antibody were diluted 1:1000 in TTBS with BSA. Blots were incubated with secondary antibody anti-mouse or anti-rabbit horse radish peroxidase conjugates diluted 1: 1000 or 1:50000 in TTBS with 3% BSA. Immunocomplexes were visualized with the Western lightning chemiluminescence kit (NEN) according to manufacturer's instructions. Immunocomplex kinase assays were performed as described previously (Menke et al., 2004).

Isolation of whole cell extracts

After harvesting and washing the cells were resuspended in extraction buffer and mechanically broken in a 60-ml Potter-Elvehjem homogenizer on ice (8 min at 1000 rpm). The homogenate was centrifuged for 30 min at 10,000 × g (Sorvall SW34 rotor). The supernatant containing the proteins had a final concentration of 9 mg/ml. The protein samples were snap frozen in liquid nitrogen and stored at −80 °C.

Digestion, desalting and purification

Samples were reduced with 10 mM DTT (Bio-rad) at 56 °C before the cysteine residues were alkylated with 55 mM iodoacetamide in the dark at RT. After dilution of the samples to a final concentration of 2 M urea with 50 mM ammonium bicarbonate, trypsin was added (Roche; 1:100 trypsin/substrate ratio). Digestion was performed overnight at 37 °C with gentle shaking. Afterwards peptides were desalted using C18 SepPak columns and dried *in vacuo.*

Immunoprecipitation and concentration of pY peptides

Peptides were resuspended in 800 µl of ice-cold IP buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% n-octyl-β-D-glucopyranoside and 1x protease inhibitor mix (Roche)) at RT for 10 min. The mixture was vortexed gently and left on a shaker for 20 minutes additionally to further dissolve the peptides. The pH of the peptides was tested and adjusted to pH 7.4. Antibody pY99 beads (Santa Cruz Biotechnology Inc) were equilibrated by adding 1ml of cold IP buffer to 48 µl of pY99 agarose beads. The beads were washed several times and the peptide mixture was added to the beads. The peptides-antibody beads mixture was incubated at 4°C overnight. Peptides were eluted from

the antibody beads with 0.15% TFA. The peptides were desalted, concentrated on stop and go C24 extraction tips prior to LC-MS.

Mass Spectrometry

Nanoscale HPLC-MS/MS experiments were performed on an Agilent 1100 nanoflow system (Agilent Technologies) connected to an LTQ-Orbitrap mass spectrometer (Thermo Electron, Fisher Scientific, Bremen Germany). Samples were loaded using an auto sampler at a flow rate of 5 µl/min on an in house packed 2-cm fused silica precolumn (100-µm inner diameter, 375-µm outer diameter, Aqua™ C18, 5 µm (Phenomenex, Torrance, CA)). Sequential elution of peptides was accomplished using a linear 3 h gradient from Solvent A (0.6% acetic acid) to 50% Solvent B (80% acetonitrile and 0.5% acetic acid) over the precolumn in line with an in house packed 40-cm resolving column (50-µm inner diameter, 375-µm outer diameter, Resprosil C18-AQ, 3 µm (Dr. Maisch, Ammerbuch, Germany). The mass spectrometer was operated in a mode to automatically switch between MS and MS/MS depending on the data. Survey of full-scan MS spectra (from m/z 300 to 1500) was acquired in the Orbitrap at a resolution of 60,000 after accumulation to a target value of 500,000. The most intense ions were sequentially isolated for accurate mass measurements and subsequently fragmented in the linear ion trap using collision-induced dissociation. The threshold for triggering an MS/MS event was set to 500 counts.

Data analysis and bioinformatics

All MS/MS spectra files from each LC-MS run were centroided and merged to a single file using Bioworks 3.3 (Thermo Electron). Runs were searched using the Mascot search engine (version 2.1.0, Matrix Science UK), against the publicly available Arabidopsis database (TAIR 8, 20080412, 32825 entries) using standard scoring. Data files were exported to an Excel format using the Rocker box tool (van den Toorn et al., 2011). Carbamidomethylation on cysteine was set as a fixed modification and oxidized methionine and phosphorylation of Ser, Thr and Tyr were set as variable modifications. Searches were done with trypsin as the proteolytic enzyme, allowing up to two miscleavages with the mass tolerance of the precursor ion set to 10 ppm, and the mass tolerance for fragment ions 0.9 Da. Only first-ranking peptide hits were allowed. Peptides were assigned to the first protein hit by Mascot. Individual MS/MS spectra from the pY phosphopeptides accepted had a Mascot score >= 20. Additionally, all identified differentially phosphorylated phosphopeptides were manually validated in each biological and technical replicate. False Discovery Rate (FDR) were calculated for data sets with different Mascot Score cut-offs by searches against a forward database and against a reversed database (Supplemental Table 2, Supporting information). For the quantitative experiment, Mascot quantification mode “15N metabolic” was chosen and peptide tolerance was initially set to 50 ppm for +2 and +3 charged peptides, and MS/MS tolerance was 0.6 Da. The mgf files were in silico recalibrated and reanalyzed with peptide tolerance set to 7 ppm, and MS/MS tolerance 0.6 Da. IsoPro 3, which makes use of the Yergey algorithm (Yergey, 1983) was used to estimate the isotope incorporation. Quantification of peptide duplets in the stable isotope labeling experiment was performed by integrating the area under the smoothed extracted ion chromatograms (XICs) of the ^{14}N and ^{15}N forms of the peptide using the monoisotopic peaks only. For LC-MS runs in which a particular peptide was not sequenced or identified, peptide duplets were matched according to accurate mass and retention time. Furthermore, ratios were averaged for peptides with and without methionine oxidation.

Data mining and analysis

149 pY peptides were searched in protein databases to examine whether any single peptide matched multiple proteins and they were mapped to 135 proteins. These proteins were subjected to a hypergeometric test of overrepresented GO Slim Plants ontology terms for *Arabidopsis thaliana* with a Benjamini & Hochberg False Discovery Rate (FDR) correction using Cytoscape v. 2.8.1 (Shannon et al., 2003) with plugin BiNGO v. 2.44 (Maere et al., 2005). Phosphorylation sites of the 135 tyrosine phosphorylated proteins were downloaded from the Arabidopsis protein phosphorylation database PhosPhAt 3.0 (http://phosphat.mpimp-golm.mpg.de) (Heazlewood et al., 2008; Durek et al., 2010). Only experimentally reported peptides with phosphotyrosine sites were included in the analysis.

Conservation of phospho tyrosine residues in plants

Multiple sequence alignments for gene families as defined by Phytozome v. 7.0 (http://www.phytozome.net) were downloaded via Biomart for core eudicot clade and the genomes of *Arabidopsis thaliana, Arabidopsis lyrata, Carica papaya, Medicago truncatula, Populus trichocarpa, Glycine max, Vitis vinifera, Mimulus guttatus, Ricinus communis, Cucumis sativus, Prunus persica, Eucalyptus grandis, Manihot esculenta* and *Citrus clementina* were included for the aligments. The position of each peptide was determined in the multiple sequence alignment and the conservation rate of the tyrosine residues was calculated. To calculate the conservation of the amino acid residues surrounding the phospho tyrosine site, the relative conservation of each of the Arabidopsis residues was calculated, and then the scores of 10 amino acids surrounding the tyrosine residue were averaged.

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ASSOCIATED CONTENT

Supporting information

Supplemental tables and figures. This material is available at http://pubs.acs.org.

CHAPTER 4

4

Phospho-protein MKKK7 is a negative regulator of PAMP-triggered immunity in Arabidopsis

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ABSTRACT

In plant signal transduction mitogen-activated protein kinases (MAPKs) are important in transducing signals from the upstream receptor to the downstream targets and they are pivotal signaling modules in pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI). In Arabidopsis, the MEK4/5-MPK3/6 module is an important part of a major MAPK cascade in immune responses. We report the functional analysis of Arabidopsis phospho-protein MAPK kinase kinase 7 (MKKK7), a MEKK-like MAPK kinase kinase (MAPKKK), as a negative regulator of PTI signaling and basal immunity. We used a loss-of-function and gain-of-function approach to demonstrate that MKKK7 modulates MPK3/6 phosphorylation, downstream defense-related gene expression and resistance to bacterial infection. Through analyses with amino acid substituted versions of MKKK7, we show that this function requires phosphorylation of MKKK7 on specific serine (Ser) residues. Our results suggest that the phosphorylation status of MKKK7 is important in the control of the regulation of the defense linked MAPKs MPK3 and MPK6, thus regulating PAMP-triggered signaling and PTI.

INTRODUCTION

Plants can respond rapidly and specifically to changing environmental conditions leading to adjustment of their metabolism and development to these changes. This requires that extracellular cues can be translated into specific intracellular responses (Fujita et al., 2006; Jones and Dangl, 2006; He et al., 2007; Schwessinger and Zipfel, 2008; Nicaise et al., 2009). During pathogen attack, plants can effectively respond by initiating various defense response mechanisms to reduce pathogen growth and restrict colonization. Initial plant defense responses rely on the host recognition of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) (Nicaise et al., 2009). Successful pathogens secrete or inject a set of effectors into the host, which suppress key steps of PAMP-triggered immunity (PTI) resulting in interference of plant defense (Cui et al., 2009). In turn plants have evolved resistance (R) proteins that monitor the targets of these effector molecules, formerly known as avirulence (Avr) factors. Perception of effector-mediated modulation of these host (R) proteins leads to a strong defense response known as effector-triggered immunity (ETI) (Schulze-Lefert and Panstruga, 2003; Jones and Dangl, 2006; Boller and He, 2009; Cui et al., 2009; Thomma et al., 2011). Type III effectors of pathogenic *Pseudomonas syringae* bacteria, such as AvrPto and AvrPtoB, act as suppressors of early defense gene transcriptional activation and block MAPK signal transduction upstream of the MAPKKKs (He et al., 2006).

ETI has initially been described as a gene-for-gene resistance model (Flor, 1971) and is often accompanied by a hypersensitive response (van Doorn and Woltering, 2005), a localized form of programmed cell death. This model elegantly illustrates the dynamic co-evolution between plants and plant pathogens (Chisholm et al., 2006; Jones and Dangl, 2006; Nicaise et al., 2009). Whereas ETI mostly monitors for pathogen attack on the inside of the cell, PTI is monitored via pattern-recognition receptors (PRR), which encode plasma membrane localized receptor-like kinases (RLKs) (Segonzac and Zipfel, 2011). The best-characterized plant PAMP-receptor is FLS2, a leucine-rich repeat (LRR) receptor kinase, that perceives a conserved 22-amino acid peptide from bacterial flagellin (flg22) and this recognition leads to the production of reactive oxygen species (ROS), apoplastic alkalinization and post-translational modifications of proteins (Felix et al., 1999; Gomez-Gomez et al., 1999; Nuhse et al., 2000), including protein phosphorylation. Protein phosphorylation/dephosphorylation changes the dynamics in the cell and the altered activity of the signaling proteins can lead to changes in protein-protein interaction or their subcellular localization (Kersten et al., 2009).

Phosphorylation is essential for the activation of the signaling module consisting of three interlinked protein kinases that form a functional MAPK cascade (Pitzschke et al., 2009; Rodriguez et al., 2010). This is one of the major eukaryotic signal transduction cascades. Sequencing of the *Arabidopsis thaliana* (Arabidopsis) genome has identified 20 MAPKs, 10 MAPK kinases (MEKs) and 80 MAPKKKs (Ichimura et al., 2002). Genomic sequences of all groups of MAPK pathway components can be found in plant species other than Arabidopsis,

including grasses, showing that these gene families evolved before bifurcation into monocot and dicot species.

The closely linked MAPK protein module consists of three subsequently acting protein kinases, a Ser threonine (Thr) MAPKKK, which phosphorylates the Ser/Thr X_{3-5} Ser/Thr motif present in the activation loop of the downstream MAPKK or MEK. The activated MEK phosphorylates the Thr and Tyr amino acid residues of the T-x-Y motif present in the activation loop of MAPKs. Phosphorylation of the MAPKs leads to the activation and subsequent phosphorylation of downstream targets in plants including WKRY transcription factors (Menke et al., 2005; Qiu et al., 2008). Although a number of downstream MAP kinase targets have been identified, the upstream events connecting PRR activation downstream to MAPK cascade activation are not known.

Tremendous progress has been made to unravel the molecular mechanisms underlying PTI and ETI, however large gaps in our understanding remain. To understand the early defense related signaling events and the changes of the responsive proteins modulated after perception of PAMPs, several large-scale phosphoproteomics approaches have been carried out (Nuhse et al., 2004; Benschop et al., 2007; Nuhse et al., 2007). In the first quantitative phosphoproteomic study, swift changes in phosphorylation of membrane-associated proteins were analyzed by $^{14}N/^{15}N$ metabolic labeling of cultured plant cells and treatment with the eubacterial PAMP flg22 and the fungal PAMP xylanase (Benschop et al., 2007). This study revealed a large set of early signaling proteins including members of the MAPKKK family. One of these proteins, named MKKK7, was dephosphorylated after flg22 treatment. The MAPKKK family comprises the largest group of MAPK pathway components, but relatively little is known about their function in plants and for only a very few MAPKKK family members an in-depth functional analysis has been performed (Frye et al., 2001; Jin et al., 2002; Champion et al., 2004). Sequence analysis of the protein kinase catalytic domain revealed that Arabidopsis MAPKKKs fall into two major subtypes: MEKKs and RAF-like kinases (Ichimura et al., 2002). Only a few members of the MEKK subfamily have been studied in more detail. Examples are Arabidopsis MEKK1, which activates MKK4 and MKK5 (Asai et al., 2002) as well as MKK1 and MKK2 (Suarez-Rodriguez et al., 2007; Gao et al., 2008) in response to flg22 perception. The orthologue of MEKK1 in *Nicotiana tabacum* (tobacco) NPK1, is involved in innate immunity and cytokinesis (Nishihama et al., 2001; Jin et al., 2002) and tobacco MAPKKKα and tomato MAPKKKε are involved in regulating cell death (del Pozo et al., 2004; Melech-Bonfil and Sessa, 2010).

There are only a few studies in the literature describing MKKK7, also known as AtMAP3Kε1, and its close paralogue MKKK6 (AtMAP3Kε2). Phylogenetic analysis clustered MKKK7 and MKKK6 as members of the cell division cycle (Cdc) family and MKKK7 was shown to be a functional homologue of fission yeast Cdc7, which is involved in cell division control (Jouannic et al., 2001). Interestingly, Cdc7 is not a MAPKKK, which could indicate that MKKK7 and its paralogue MKKK6 might also have a different function than MAPKKKs. Chaiwongsar et al. (2006) showed that double homozygous mutant plants lacking both MKKK7 and MKKK6 are male sterile caused by pollen lethality. They also determined that

double-mutant pollen grains develop plasma membrane irregularities following pollen mitosis I. Analysis of the MKKK7:YFP fusion protein indicated that a substantial portion of MKKK7 is localized on the plasma membrane. These results suggest that MKKK7 is required in Arabidopsis pollen viability in the absence of the closest homologue MKKK6. In a recent study in tomato, a related MAPKKKε was found to function in plant innate immunity and to regulate cell-death (Melech-Bonfil and Sessa, 2010). Although the tomato MAPKKKε and MKKK7 are close homologues based on amino acid sequences, it remains to be determined whether they are functional orthologues, since tomato has only one MAPKKKε.

Our previous work has identified Arabidopsis MKKK7 as a membrane associated phospho-protein. Here we report on the functional analysis of this protein and we implicate this protein as a negative regulator of PTI signaling and basal immunity in Arabidopsis. Through using amino acid substitution mutants we show that two of the four phosphorylated Ser residues of MKKK7 are important for its function.

RESULTS

Phospho-protein MKKK7 is modulated upon PAMP perception

Using a quantitative phosphoproteomics approach, we previously identified a large number of differentially phosphorylated sites of membrane-associated Arabidopsis proteins in response to PAMP-treatment (Benschop et al., 2007). We selected a set of 4 differentially phosphorylated MAPKKKs and one MAP4K for in-depth analysis to investigate their possible role in PAMP-triggered signal transduction and downstream PTI. Here, we describe the functional analysis of one of these kinases, MKKK7 (At3g13530), a MAPKKK with a typical serine-threonine kinase domain. Based on the amino acid sequence of the kinase catalytic domain, MKKK7 was classified as a member of the subfamily of the MEKKs, subgroup A4 (Ichimura et al., 2002). The MKKK7 protein was isolated as a membrane-associated protein (Benschop et al., 2007) and was previously shown to be localized in the plasma membrane (Chaiwongsar et al., 2006).

We identified 4 phosphorylated Ser residues in MKKK7 (Fig.1A and 1B; Benschop et al., 2007). And in response to flg22 induction, this protein was differentially (de) phosphorylated on Ser^{452} residue (0.7 fold) and Ser^{854} residue (0.6 fold), while the phosphorylation of the other two residues (1.0 fold) was not affected by flg22 treatment (Fig. 1A; Benschop et al., 2007). The location of the identified phosphorylated Ser residues is outside of the kinase domain, which is located at the N-terminal half of the protein. The differentially phosphorylated Ser residues are both located within the central domain of the protein, but outside of any recognizable protein domain. The phosphorylated Ser sites are located in a region flanking armadillo (ARM)/HEAT repeat domains found in MKKK7 and homologous MAPKKKs in other plant species.

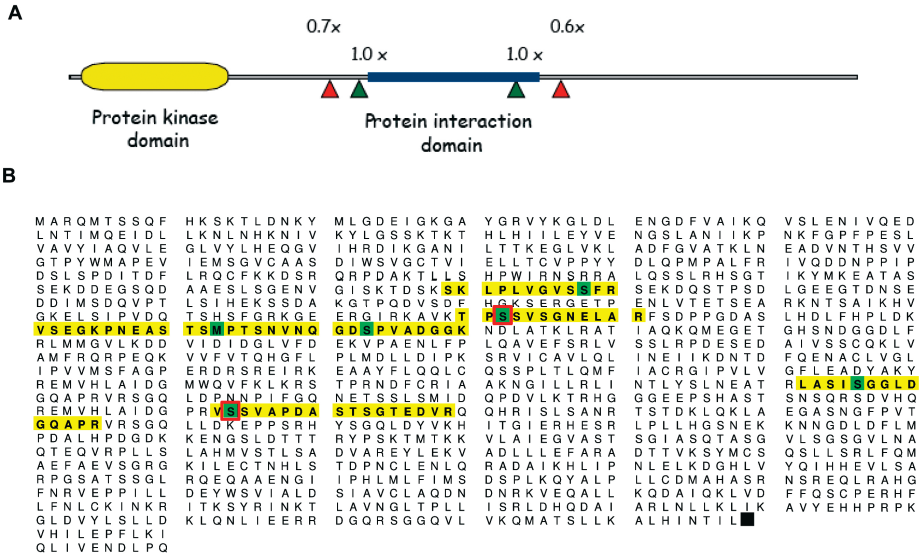

Figure 1. **Differential phosphorylation of MKKK7. A)** Protein structure of MKKK7 with the protein kinase domain shown in yellow and an ARM repeat domain shown in blue. The position of the phosphorylated Serine residues after flg22 treatment is shown with triangles. The green triangles indicate non-differentially phosphorylated sites and the red triangles indicate differentially phosphorylated serine sites. Respective ratios are indicated above the protein. **B)** Protein sequence of MKKK7, highlighted in yellow are all (phospho)peptides measured by mass spectrometry. Highlighted in green are modified residues and the red box around the serine residues indicates differential phosphorylation after flg22 treatment.

MKKK7 is annotated as a gene on chromosome 3 with a length of 7387 nucleotides and encoding a protein sequence of 1368 amino acids. It has a complex structure which consists of 24 exons and 23 introns (Fig. 2A). The complete protein sequence was compared using the BlastP algorithm (http://blast.ncbi.nlm.nih.gov) in order to find closely related homologues. MKKK7 shares 87% homology with the Arabidopsis paralogue MKKK6, and 72% homology with a homologue in tomato annotated as SlMAPKKKε (data not shown). Its kinase domain is conserved in higher plant species, including close relatives, such as *Brassica napus* and the more distantly related species *Medicago truncatula* and *Vitis vinifera.* Furthermore, close homologues with high levels of conservation spanning the entire length of the protein can be found in most, but not all, sequenced dicot species and also in more distantly related monocot species such *Oryza sativa.* Based on this high level of sequence conservation it is likely that the phospho-protein MKKK7 and its orthologues have an evolutionary conserved function in plant signal transduction.

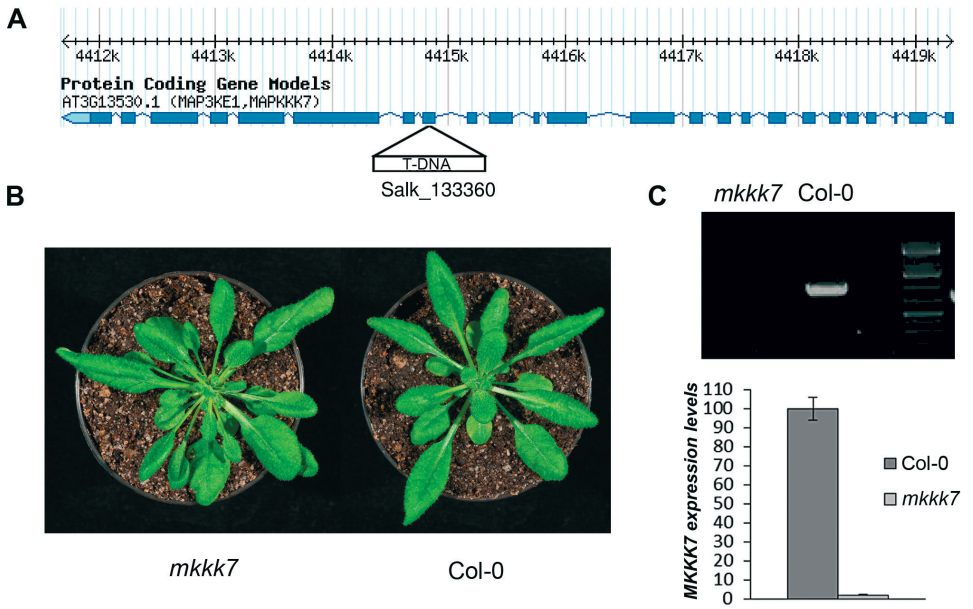

Figure 2. Characterization of the *mkkk7* loss-of-function mutant. **A)** Intron-exon structure according to TAIR and the position of the T-DNA insertion in chromosome 3. **B)** Five-week-old *mkkk7* and Col-0 plants, grown in short day conditions. **C)** The T-DNA insertion site was verified with gDNA specific primers for *MKKK7* in Col-0 and *mkkk7* homozygous insertion line (top panel). QRT-PCR with gene-specific *MKKK7* primers for Col-0 and *mkkk7* (bottom panel). Ct values were normalized to *Tubulin*. Results shown are means ± SEM (*n*=3). Experiments were repeated three times with similar results.

Silencing of *MKKK7* doesn't cause a phenotype above ground

To examine the function of MKKK7 in flg22-mediated defense responses, we obtained T-DNA insertion lines. Mutant alleles of *MKKK7* in the Col-0 background were obtained for three independent T-DNA insertion lines from the Salk Institute (Alonso et al., 2003), but only one of these T-DNA insertions (Salk_133360) could be verified. The T-DNA insertion in Salk_133360 is in annotated exon 17 and was confirmed by PCR with gene specific primers (Fig. 2C, top panel). In line with the fact that this *mkkk7* mutant line is homozygous for the T-DNA insertion, quantitative RT-PCR (qRT-PCR) analysis revealed that there is a significant reduction in the endogenous transcript levels of the *MKKK7* gene, making this a knock-down mutant and not a null-mutant (Fig. 2C, bottom panel). We designated this T-DNA insertion mutant *mkkk7-1.*

Although MKKK7 is a functional homologue of MKKK6 it was not possible to generate a viable *mkkk6 mkkk7* double mutant line, that is described as pollen lethal (Chaiwongsar et al., 2006). When *mkkk7* was grown at temperatures typical for Arabidopsis propagation (22-24°C), it resembled the Col-0 wild-type (Fig. 2B) and had no obvious growth or flowering phenotype. Under normal growing conditions seeds of these plants also

germinated normally in both long-day and short-day growth conditions (data not shown). We did notice a small difference in growth during the first two weeks after germination, resulting in shorter roots in *mkkk7* and a slight reduction in seedling size when grown on agar solidified medium (data not shown). Upon transplantation into soil the above ground parts recovered quickly resulting in wild-type looking plants. Thus, the knock-down mutant *mkkk7* has no obvious growth phenotype in the above-ground plant parts.

***MKKK7* negatively regulates flg22-induced growth inhibition**

Bacterial flagellin acts as a potent elicitor of defense responses in cell cultures of Col-0 (Felix et al., 1999; Benschop et al., 2007) and as a result inhibits growth of Arabidopsis seedlings (Gomez-Gomez et al., 1999). Treatment with flg22 affects growth of roots, leaves and cotyledons and results ultimately in a reduction in fresh weight. To test whether growth inhibition was altered in *mkkk7*, seedlings of Col-0, *mkkk7* and the flagellin receptor mutant *fls2* were grown in liquid ½ MS medium before transferring them into ½ MS medium with an increasing concentration of the peptide flg22. A dose range of 10 to 200 nM flg22 affected the growth of Col-0 and resulted in a similar decrease in overall growth of *mkkk7* as compared to the seedlings growing without flg22 (Fig. S1). The growth of *mkkk7* seedlings was slightly more inhibited compared to Col-0 in five independent experiments, but was statistically not significantly different, whereas *fls2* growth was unaffected by the flg22 treatment.

We next analyzed flg22-inhibited root growth separately, since flg22 was previously reported to inhibit root growth more strongly than total biomass accumulation (Ranf et al., 2011). We tested Col-0, *mkkk7* and two independent estradiol-inducible overexpression lines of *MKKK7* fused to *GFP* (*ind-MKKK7*). Seedlings were grown on agar-solidified medium containing 2 µM estradiol and varying concentrations of flg22. At 100 nM flg22, the lowest flg22 concentration tested, the length of the primary root was inhibited in Col-0 to 90% relative to the mock treatment, but this was not statistically significant. In the *mkkk7* mutant, no flg22-mediated inhibition of root growth was observed (Fig. 3). At a 10-fold higher concentration, flg22 inhibited root growth was nearly equal in Col-0 and *mkkk7* to a level of 87% and 83 % of the mock treatment respectively.

We also tested primary root length inhibition in response to a different PAMP (elf18), that is perceived via a different receptor (EFR), but induces a similar downstream PTI signaling pathway (Nicaise et al., 2009). Comparable, but more pronounced results were obtained for elf18-inhibited root growth, with *mkkk7* and Col-0 showing identical inhibition at both 100 nM and 1 µM elf18 (Fig. 3). It should be noted that the primary root of the *mkkk7* mutant line was shorter under all tested conditions (Fig. S2). Similarly, we also noted that the shoot was smaller and responded more strongly to PAMP-triggered growth inhibition as compared the Col-0 control (Fig. S2). These results are in line with the observations made with flg22-inhibited growth responses of whole seedlings in liquid medium and indicate that *mkkk7* growth is slightly more inhibited in response to flg22.

If loss of *MKKK7* makes seedlings more responsive to flg22-induced growth inhibition, then enhancing *MKKK7* levels should make seedlings less sensitive. Indeed, inducible overexpression of *MKKK7:GFP* resulted in insensitivity to flg22-mediated growth inhibition at the lower as well as the higher concentrations in both *ind-MKKK7 L13* and *ind-MKKK7 L14* lines (Fig. 3). Interestingly, these *MKKK7* overexpressing lines were still responsive to 100 nM and 1 µM elf18, as shown by the significant reduction in primary root growth (Fig. 3). Taken together these results suggest that *MKKK7* negatively regulates flg22-triggered growth inhibition and that the reduced primary root length of *mkkk7* seedlings in the absence of PAMP-perception phenocopies low level flg22-perception. Furthermore, this response is specific for FLS2-mediated signaling, as elf18-induced growth inhibition, mediated via EFR signaling, is not modulated by varying *MKKK7* levels.

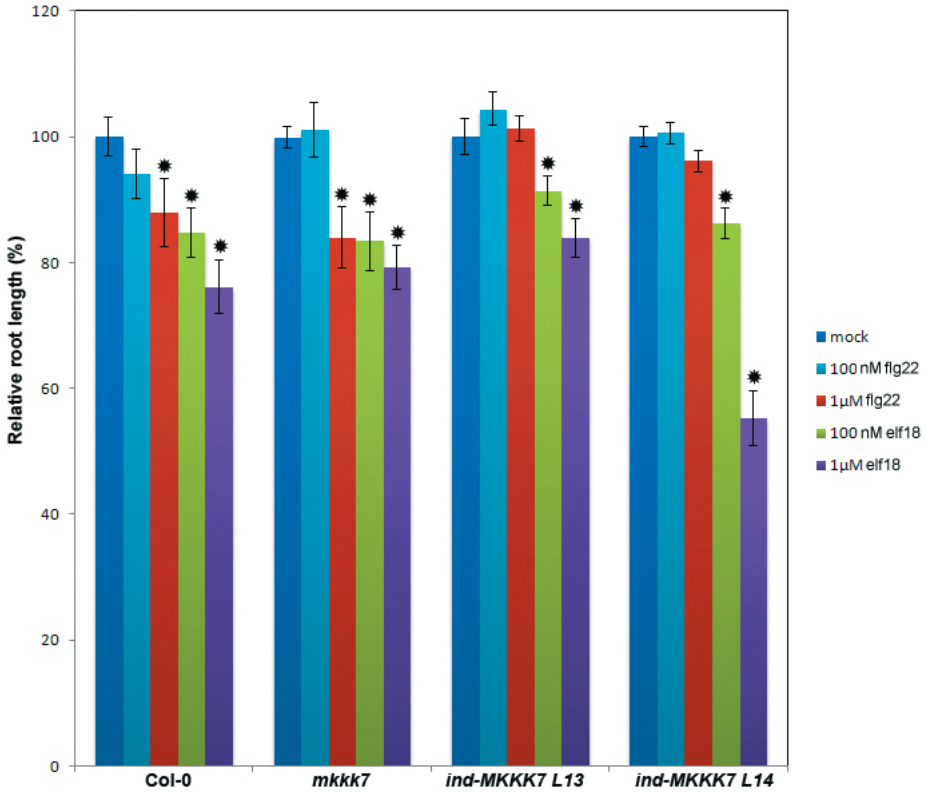

Figure 3. Flagellin-induced root growth inhibition is suppressed by MKKK7. Dose-dependent inhibition of primary root growth by the PAMPs flg22 and elf18. Five-day-old seedlings were transferred to agar solidified ½ MS medium with 2 µM estradiol at indicated concentrations of flg22 or elf18 and the length of the primary root was recorded 8 to 9 days later. Primary root length was measured with ImageJ software and compared relative to the mock treated plants, which was set at 100%. Data represents mean values ± SEM (n=10; *, P<0.05; paired t-test). Experiment was repeated twice with similar results.

MKKK7 represses flg22-induced MAPK activation

To investigate whether MKKK7 is involved in the modulation of PTI downstream of FLS2, we next tested the activation of downstream MAPKs in seedlings with altered levels of *MKKK7* expression. Since the activation status of MAPKs MPK3, MPK4 and MPK6 correlates very well with the phosphorylation of the activation loop, we used an antibody raised against the doubly phosphorylated activation loop of mammalian ERK1/ERK2 protein (anti-p42/p44) to detect changes in MAPK phosphorylation and activation. This antibody also recognizes dually phosphorylated residues in the T-x-Y motif in the activation loop of Arabidopsis MAPKs (Anderson et al., 2011; Mithoe et al., 2012). Seedlings were incubated for a short time frame of 0, 10 or 30 min with 1 µM flg22 to focus on early defense signaling in Col-0 and in *mkkk7*.

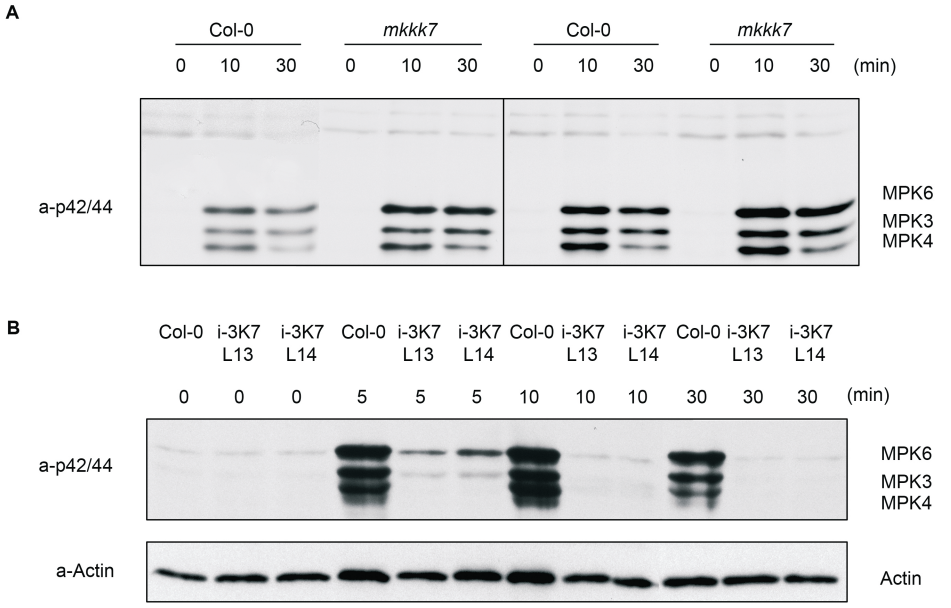

Figure 4. Flg22-induced MAPK phosphorylation is inhibited by MKKK7. A) Immunoblot analysis of MAPK phosphorylation after flg22 induction in Col-0 and in *mkkk7* seedlings. Protein extracts were made of seedlings treated with flg22 and samples were taken at t=0, 10 and 30 min post induction. The p42/44 antibody was used to detect phosphorylated MAPKs. Position of the individual phosphorylated MAPKs is indicated at the right. Equal loading of protein is indicated by an aspecific reactive protein band on the top of the panel. Right and left panel show independent biological replicates. **B)** Immunoblot analysis of MAPK phosphorylation after flg22 induction in Col-0 and *ind-MKKK7* seedlings. Protein extracts were made of seedlings treated with flg22 and samples were taken at t=0, 5, 10 and 30 min post induction. The p42/44 antibody was used to detect phosphorylated MAPKs. Position of the individual phosphorylated MAPKs is indicated at the right (top panel). α-Actin antibody was used as a loading control (bottom panel). i-3K7 L13, *ind-MKKK7* line13; i-3K7 L14, *ind-MKKK7* line14.

Kinase activity of three different MAPK family members was detected in the Col-0 wild type and also in *mkkk7* (Fig. 4A). As shown in two independent biological replicates (left and right panel of Fig. 4A), MPK4 (42 kD) and MPK3 (44 kD) were phosphorylated on the T-x-Y motif in Col-0 and *mkkk7* after treatment with flg22. There were minor differences observed in MPK4 and MPK3 phosphorylation in *mkkk7* in the observed time frame, with slightly higher phosphorylation in *mkkk7* at 10 min after induction with flg22. MPK6 (49 kD), was however clearly differentially phosphorylated in Col-0 and *mkkk7*. It appears that the activation loop of MPK6 is phosphorylated to a higher level in the *mkkk7* mutant as compared to Col-0 at both 10 and 30 min after induction with flg22. We observed this enhanced MPK6 phosphorylation in three independent biological replicates. Equal loading of the blots was judged based on an aspecific reactive high molecular weight band (Fig. 4A, top of the panel). Expression of *MKKK7* was induced in seedlings of two independent *ind-MKKK7* lines 24 h prior to stimulation with flg22. Basal levels of MPK6 phosphorylation could be detected in Col-0 and both *ind-MKKK7* lines at a similar level prior to induction with flg22, indicating that the MPK6 phospho-protein levels were equal before induction (Fig. 4B). The addition of 1 µM flg22 rapidly and strongly induced MPK3, MPK4 and MPK6 phosphorylation at 5 and 10 min and with reduced levels at 30 min in the Col-0 seedlings (Fig. 4B upper panel). In both *ind-MKKK7* lines the flg22-induced phosphorylation of MPK3 and MPK6 was detected at 5 min at a dramatically lower level than in Col-0, while MPK4 phosphorylation was completely undetectable. Moreover, at 10 and 30 min induction the level of phosphorylation of all three MAPKs was completely suppressed to basal level in these seedlings. Similar inhibition of MAPK phosphorylation was observed in three independent biological replicas for both transgenic lines. Equal loading of protein was verified by reprobing the blots with an α-Actin antibody (Fig. 4B lower panel). These data show that overexpression of *MKKK7* suppresses MPK3, MPK4 and MPK6 phosphorylation and activation in response to flg22 induction, but that these seedlings are still capable of flg22 perception through FLS2. The combined results from both the loss-of-function and the gain-of-function experiments demonstrate that MKKK7 negatively regulates flg22-triggered MAPK phosphorylation downstream of the FLS2 receptor.

MKKK7 represses defense gene expression downstream of the MPK3/MPK6 MAPK cascade

In mesophyll protoplasts, induction with flg22 leads to the activation of Arabidopsis MPK3 and MPK6 upstream of *WKRY29* and *FRK1* expression (Asai et al., 2002). We used flg22-stimulated *WRKY29* and *FRK1* expression to connect MKKK7 modulated MAPK activity with downstream defense gene expression. First we looked at gene expression levels of *WRKY29* and *FRK1* in mesophyll protoplasts of *mkkk7*. We used the promoters of transcription factor *WKRY29* and receptor-like kinase *FRK1* fused to the firefly luciferase reporter (fLUC) reporter (Asai et al., 2002) and transiently expressed these constructs in mesophyll protoplasts from Col-0 or *mkkk7* plants. Treatment of protoplasts with flg22 activated the *WKRY29* and

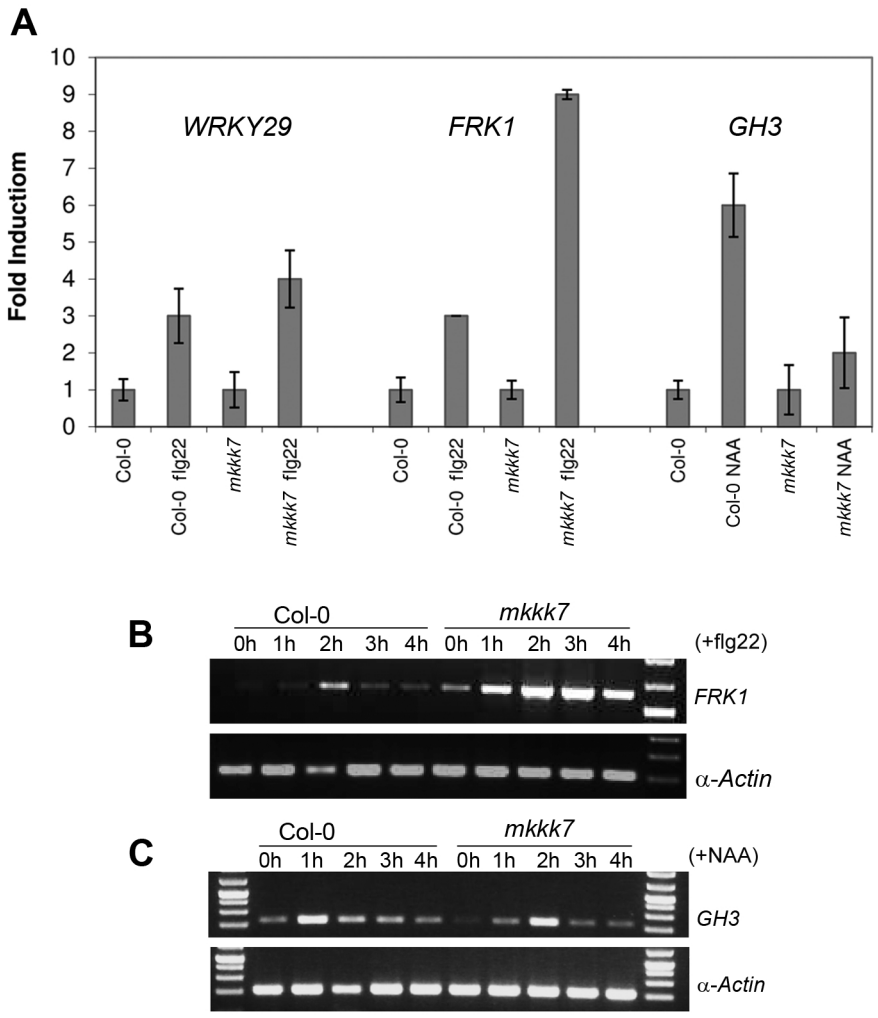

Figure 5. Enhanced flg22-induced defense gene expression in *mkkk7* mutant. A) Transient expression analysis in Arabidopsis mesophyll protoplasts shows enhanced defense gene expression in *mkkk7* protoplasts after flg22 treatment and reduced auxin responsive gene expression as compared to Col-0 protoplasts. Protoplasts were isolated from 4 week old plants and transfected with *pWKRY29::fLUC (WRKY29)*, *pFRK1::fLUC(FRK1)* or *pGH3::fLUC (GH3)* constructs together with *35S::rLUC*, as indicated in the graph. Protoplasts were treated with either 10 µM flg22, 8 µM auxin (NAA) or mock treated as indicated. All measurements were normalized to the *rLUC* activity and expression levels were calculated relative to the mock treated control sample as shown as fold induction. Results shown are means ± SEM (*n*=2). This experiment was repeated 6 times with similar results. **B)** RT-PCR analysis of flg22-activated *FRK1* and *α-Actin* (control) transcripts in leafs. Leaf strips of Col-0 and *mkkk7* were treated with 10 µM flg22 for t=0, 1, 2, 3 and 4 h. **C)** RT-PCR analysis of auxin-responsive *GH3-like (GH3)* and *α-Actin*(control) transcripts in Col-0 and *mkkk7* leafs. Arabidopsis leaf strips were treated with 8 µM NAA for t=0, 1, 2, 3 and 4 h. For each RT-PCR experiment biological replicates were done showing the same trend.

FRK1 promoter expression in Col-0 protoplasts (Fig. 5A). In *mkkk7* protoplasts the activity was induced to a higher level after treatment with flg22 for both the *WRKY29* and *FRK1* promoters (Fig. 5A).

To verify that these changes in gene expression were related to FLS2 signaling and not caused by a more general enhancement of gene expression, we evaluated the expression of the auxin-responsive promoter of the *GH3* gene (Kovtun et al., 2000). The protoplasts were treated with the synthetic auxin 1-naphthaleneacetic acid (NAA) and in Col-0 protoplasts the *GH3* promoter activity was increased (Fig. 5A). However, in *mkkk7* protoplasts there was reduced *GH3* promoter activation after induction with NAA. These observations indicate that enhanced *WRKY29* and *FRK1* gene activation in *mkkk7* protoplasts is not due to general enhancement of induced gene expression and additionally indicate a reduction of auxin responsiveness in *mkkk7* plants.

The observations in mesophyll protoplasts, were confirmed by RT-PCR analysis of *WRKY29* (data not shown) and *FRK1* mRNA levels in leaf strips treated with flg22. The results show an increase in mRNA levels of the *FRK1* gene in Col-0, 2 h after treating leaf strips with flg22, followed by a decrease in *FRK1* gene transcription. In *mkkk7*, basal *FRK1* gene expression is enhanced and after flg22 treatment the mRNA levels of the *FRK1* gene increased to a higher level relative to Col-0. Even at 4 h after treatment there is more abundant *FRK1* expression in *mkkk7* (Fig. 5B, upper panel), indicating sustained defense gene activation in *mkkk7* leaf strips. The endogenous control gene *Actin* showed unaltered expression levels (Fig. 5B, lower panel). Taken together our results suggest that loss of MKKK7 activity enhances early defense gene expression, through derepression of a MAPK cascade which includes MPK3 and MPK6.

We also verified the differences in *GH3* gene expression by RT-PCR analysis of leaf strips treated with NAA. Transcription of the *GH3* gene was increased in Col-0 at 1 h after induction with NAA, while in *mkkk7* an increase of gene transcription was delayed until 2 h after induction with NAA (Fig. 5C upper panel). No changes were observed in *Actin* gene expression (Fig. 5C lower panel). The time course demonstrates that the increase in *GH3* mRNA levels in Col-0 is overall higher compared to the transient increase seen in *mkkk7*. Whether this is indicative of overall lower auxin responsiveness in *mkkk7* remains to be determined.

Phosphorylation of MKKK7 is required for maximum repression of flg22-induced defense gene expression

Through a loss-of-function approach we demonstrated enhanced MAPK activity and defense gene expression when MKKK7 protein levels are lowered. To complement these results we used the protoplast transient expression assay to evaluate the effect of *MKKK7* overexpression on flg22-triggered gene expression. This assay is also suited to test the effect of overexpression of point mutated versions of *MKKK7*. We used site-directed mutagenesis to change the two previously identified Ser residues of MKKK7 (Benschop et al., 2007) into

alanine (Ala/A) or aspartate (Asp/D). Replacing Ser with Ala creates a non-phosphorylatable version, and replacing Ser with Asp often creates a phosphomimic version at the replaced residue. The full length *MKKK7* coding sequence with wild-type sequence, a double Ala substituted version (*MKKK7*^{AA}) and a double Asp substituted version (*MKKK7*^{DD}) were cloned behind the constitutively active *35S* promoter (Fig. 6A). These constructs were transiently expressed in protoplasts as effector constructs together with the previously described reporter construct combination.

Col-0 protoplasts were transformed with a negative control effector construct (*35S::GFP*). The flg22-induced increase in the promoter activity of *WKRY29* was 3-fold (Fig. 6B). Overexpressing *MKKK7* shows no increase of the *WKRY29* promoter activity after flg22 stimulation, suggesting that the FLS2-dependent cascade was not responsive. The activity of *WKRY29* even decreased in comparison to the untreated sample, possibly indicating that basal signaling and expression are blocked at elevated levels of *MKKK7* expression (Fig. 6B). Overexpression of the mutated non-phosphorylatable mutant (*35S::MKKK7*^{AA}) in the protoplasts shows an increase in *WRKY29* promoter activity almost equal to the negative control transformed protoplasts (Fig. 6B). When the *MKKK7*^{DD} phosphomimic version was expressed this resulted in loss of flg22-responsive *WRKY29* gene expression and a decrease in basal *WRKY29* promoter activity similar to that observed in *MKKK7* overexpressing cells. Experiments with the *FRK1* reporter construct gave similar results (Fig. S4). Overexpression of the complete cDNA of *MKKK7* or Asp substituted *MKKK7*^{DD} thus resulted in blocking the flg22-induced early defense gene expression. This indicates that phosphorylation of MKKK7 is required for suppression of flg22-induced defense gene expression. Furthermore, since *WRKY29* and *FRK1* expression in response to flg22 requires MPK3 and MPK6 activity, we infer that phosphorylation of MKKK7 is required to suppress MAPK activation upstream of defense gene expression.

MKKK7 overexpression causes growth defects and stress symptoms

The combined data obtained with the *mkkk7* mutant and the transient overexpression of *MKKK7* showed that MKKK7 suppresses MAP kinase activity and early defense gene expression. To test whether these observations could be extended to changes in disease resistance we made constructs to complement *mkkk7,* as well as to create stable *MKKK7* overexpression lines. Two types of constructs were made using the coding region of *MKKK7* fused with *GFP* under the control of two different promoters. These included the native *MKKK7* promoter for complementation and the *35S* promoter for overexpressing *MKKK7* (Table S3). We transformed these different constructs into the *mkkk7* background and into Col-0. Phenotypes of the T1 and T2 generation of Norf-resistant transformants were evaluated (Fig. 7A-F). Based on the available information from *pMKKK7:GUS* lines, *MKKK7* is expressed in the leaf margins, trichomes, root tips and in developing ovules (Bedhomme et al., 2009). Complementation with *MKKK7:GFP* under the control of the native promoter in *mkkk7* and Col-0 background appeared normal with a wild-type phenotype above

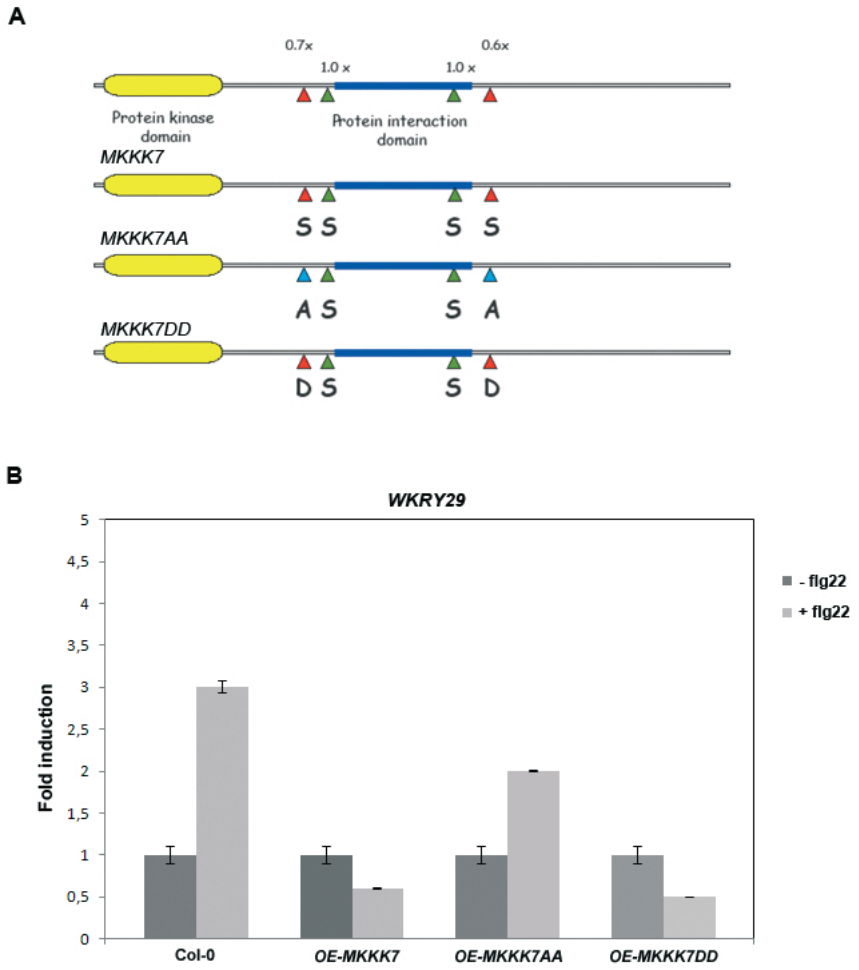

Figure 6. Phosphorylation of MKKK7 is required to suppress flg22-induced *WRKY29* gene expression. A) Protein structure of MKKK7 and mutated versions of MKKK7 with the protein kinase domain shown in yellow and an ARM repeat domain shown in blue. The position of the phosphorylated Ser residues after flg22 treatment is shown with triangles and bold S below the protein. The green triangles indicate non-differentially phosphorylated sites and the red triangles indicate differentially phosphorylated serine sites. Respective ratios are indicated above the protein. Amino acid substitute versions of MKKK7 are shown below the wild-type. S, serine; A, alanine; D, aspartate. **B)** Transient co-expression of *MKKK7* inArabidopsis mesophyll protoplasts shows suppression of *WRKY29* gene expression after flg22 treatment with 10 µM flg22. Protoplasts were transfected with *pWKRY29::fLUC*, *35S::rLUC* and indicated overexpression constructs of *MKKK7* (*OE-MKKK7, OE-MKKK7^{AA} or OE-MKKK7^{DD}*) as indicated at the right of the graph. All measurements were normalized to the rLUC activity and expression is relative to the mock treated control sample, shown as fold induction. Results shown are means ± SEM (*n*=2). Experiment was repeated twice with similar results.

ground. Expressing *pMKKK7::MKKK7:GFP* constructs in *mkkk7* also complemented the root phenotype (chapter 5) and the seeds of these plants also showed normal germination. Overexpressing *MKKK7* in the *mkkk7* background resulted in plants with a mostly wild-type phenotype, producing slightly shorter petioles and mildly different leaf morphology (Fig. 7C). However, overexpressing *MKKK7* in Col-0 resulted in a range of phenotypes and seedlings of T2 lines had very severe phenotypes. The observed phenotypes ranged from generally effects in development, such as aberrant leaf morphology and reduced stature to stressed dwarf plants with dark green 'cactus-like' leaves with closely spaced trichomes (Fig. 7B, 7D left panel, Fig. 7F). When the leaves were observed in more detail they showed necrotic lesions and what appeared as a HR-like response leading to cell death as well as chlorosis (Fig. 7F).

QRT-PCR revealed a 200-fold increase in *MKKK7* mRNA levels in the *OE-MKKK7* lines in the Col-0 background, and a 50-fold increase in the *mkkk7* background (Fig. 7E). It is likely that since *MKKK7* is expressed at very low levels in the plant, sustained expression at levels much higher than endogenous levels causes the observed growth defect phenotype with senescence and HR. We observed that the more extreme *35S::MKKK7:GFP* lines often showed signs of spontaneous infection with unidentified pathogens and frequently died before flowering (data not shown). Therefore the observed necrosis and chlorosis could also have been a direct result of spontaneous infection of these lines and indirectly caused by *MKKK7* overexpression.

MKKK7 represses PAMP-triggered immune response

The spontaneous infection of overexpression lines, enhanced defense signaling and defense gene expression in the loss-of-function mutant lines indicate that MKKK7 negatively regulates basal immunity. To gain insight into whether MKKK7 regulates immune responses in Arabidopsis, we first studied basal immunity in *mkkk7* as compared to Col-0. We used a bioassay in which Col-0 and *mkkk7* plants were dipped into a suspension of virulent *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) bacteria and disease symptoms, including water soaked lesions and chlorosis on leafs (Fig. 8A and 8B) were scored 2 to 4 days after inoculation (dpi). At 3 dpi with *Pst* DC3000 the percentage of leaves showing disease symptoms was significantly less in *mkkk7* compared to Col-0 (Fig. 8A). We repeated these experiments 3 times with similar results. It appears that *mkkk7* is less susceptible to this virulent pathogen as judged by symptom development and suggests that MKKK7 can indeed suppress basal immune responses in Arabidopsis.

To distinguish between delayed disease symptom development in *mkkk7* and actual enhanced resistance to virulent *Pst* DC3000 we quantified bacterial growth in the loss-of- function mutant *mkkk7* and the gain-of-function transgenic line carrying construct *35S::MKKK7:GFP* in the *mkkk7* background. At 3 dpi with virulent *Pst* DC3000, the bacterial titer in leaves of *mkkk7* was significantly lower as compared to Col-0 and *OE-MKKK7* (Fig. 9A). No large effect of constitutive overexpression of *MKKK7* in *OE-MKKK7* on growth of

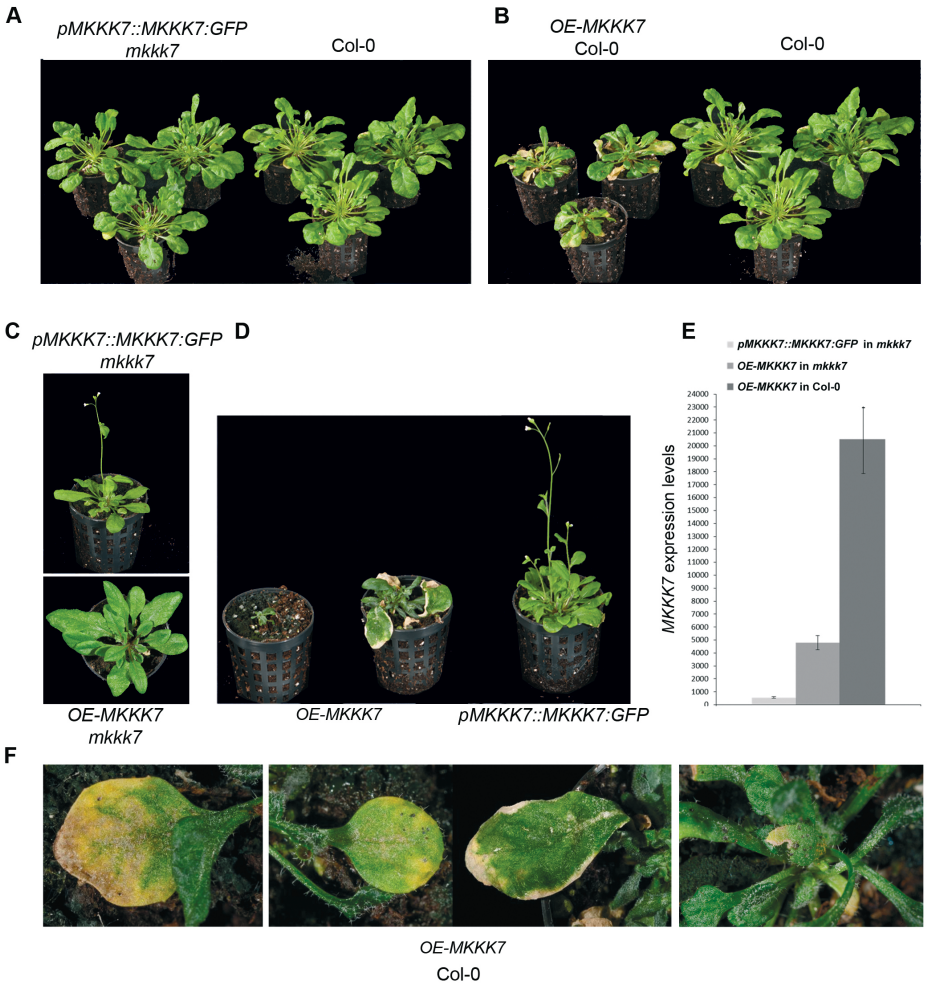

Figure 7. Phenotypes and characterization of *MKKK7* overexpressing lines. A) Expression of *MKKK7:GFP* under the expression of the endogenous *MKKK7* promoter results in wild-type looking plants at 6 weeks old. **B)** Overexpression of *MKKK7:GFP* (*OE-MKKK7*) under the control of the *35S* promoter causes stressed plants with early senescence; shown are T2 transgenic lines with intermediate phenotypes at 6 weeks old. **C)** Examples of *pMKKK7::MKKK7:GFP* and *35S::MKKK7:GFP* in the *mkkk7* background at 4 weeks old. **D)** Examples of strong and intermediate overexpression phenotypes of *35S::MKKK7:GFP* (*OE-MKKK7*) in Col-0 background as compared to *pMKKK7::MKKK7:GFP* in Col-0 background. A range of phenotypes from small dark green plants to plants with aberrant leaf morphology were observed. Examples shown are T2 transgenic lines at 5-6 weeks old. **E)** Quantification of expression levels of *MKKK7* transgenes in different transgenic lines by qRT-PCR. Results shown are means ± SEM (*n*=2). **F).** Close-up of leafs of *OE-MKKK7* seedlings; leafs are showing chlorosis (all panels), HR-like cell death (middle panel) and senescence (middle and right panel) as well as aberrant morphology (all panels) and dark green leafs with dense trichomes (middle and right panel).

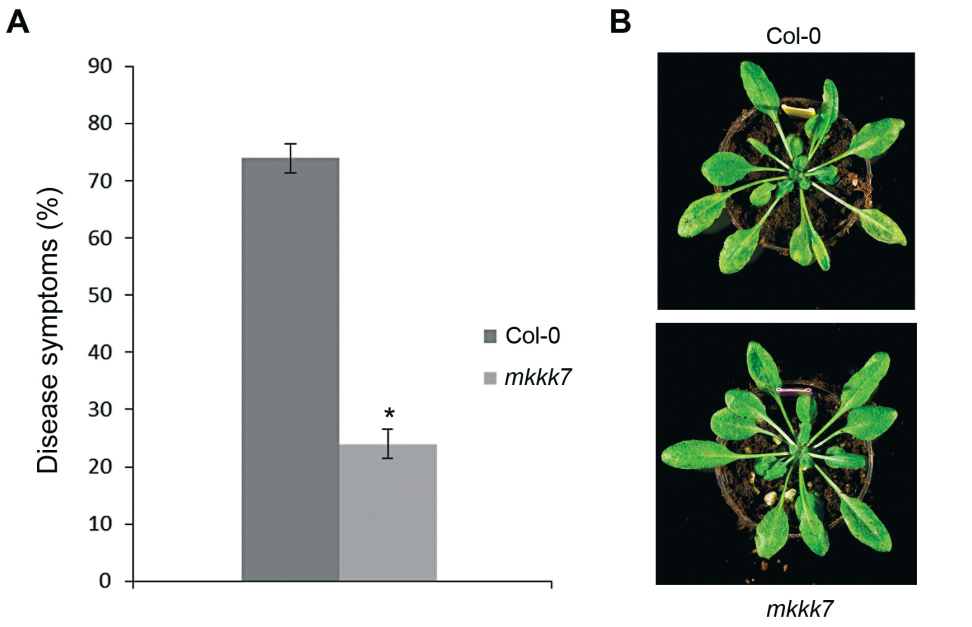

Figure 8. Effect of MKKK7 on resistance against *Pst* DC3000. A) Four-week-old seedlings were dipped into a suspension containing virulent *Pst* DC3000 and 72 h later the disease symptoms were scored. Data represents mean values ± SEM (*n*=20; *, P<0.05; paired t-test). Three biological experiments were done showing similar results. **B)** Examples of disease symptoms such as water soaked lesions and chlorosis are clearly visible on several leaves of Col-0 at 72 h after inoculation (top panel) whereas fewer symptoms have developed on inoculated *mkkk7* plants (lower panel).

Pst DC3000 could be observed. However, based on symptom development, overexpressing *MKKK7* in the mutant background did enhance the susceptibility to *Pst* DC3000 infection (Fig. 9B). These results are in support of the notion that the decrease in disease symptoms observed in the infected *mkkk7* is caused by more effective restriction of bacterial growth and that MKKK7 is a suppressor of basal immunity. To further substantiate this finding we made use of stable *MKKK7* overexpressor lines under control of an inducible promoter (see next paragraph).

Phosphorylation of MKKK7 is necessary for suppression of basal immunity

Overexpression of the *MKKK7* full length coding region and the *MKKK7* construct with the double dephosphorylated Ser mutated to Asp in protoplasts resulted in a complete shut-down of the flagellin-triggered defense gene expression, while overexpression of the Ser to Ala substituted version of MKKK7 did not show suppression (Fig. 6B and S4). To gain insight in the importance of phosphorylation of MKKK7 in suppression of basal immunity we made transgenic lines expressing $MKKK7^{AA}$ and $MKKK7^{DD}$. Since overexpression of *MKKK7* resulted in a spectrum of phenotypes and the severe phenotype was highly susceptible for

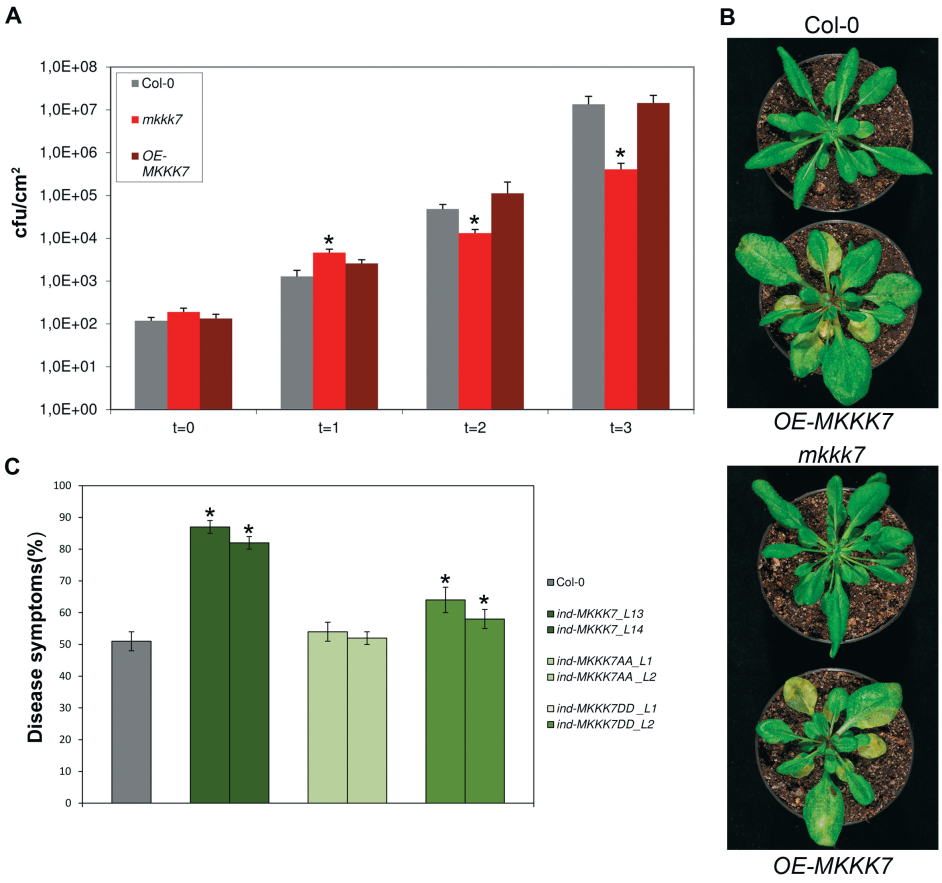

Figure 9. *MKKK7* expression levels determine susceptibility to virulent bacterial infection. A) Quantification of bacterial virulence in Arabidopsis lines Col-0, *mkkk7* and *OE-MKKK7* in the *mkkk7* background. Four to five-week-old plants were pressure-infiltrated with virulent *Pst* DC3000 and at indicated time points triplicate samples were harvested and bacteria re-isolated on selective media. The number of colony forming units (cfu/cm²) was determined at 0, 1, 2 and 3 days post inoculation (dpi). Data represents mean values ± SEM (*n*=20; *, P<0.05; paired t-test). The experiments were done twice with similar results. **B)** Examples of symptom development at 2 dpi in the *OE-MKKK7* in *mkkk7* background as compared to Col-0 (top panel) and *mkkk7* (lower panel). **C)** Disease symptom development in *Pst* DC3000-infected lines with estradiol inducible constructs of *ind-MKKK7* L13, *ind-MKKK7* L14, *ind-MKKK7^{AA}* L1, *ind-MKKK7^{AA}* L2, *ind-MKKK7^{DD}* L1 and *ind-MKKK7^{DD}* L2. Two independent transgenic lines for each construct were grown under short-day conditions and disease symptoms were scored 3 dpi. Data represents mean values ± SEM (*n*=20; *, P<0.05; paired t-test). This experiment was repeated with a similar outcome.

spontaneous infections under normal growth conditions, we choose to use an inducible version of amino acid substituted *MKKK7* (*ind-MKKK7^{AA}*, *ind-MKKK7^{DD}*). To allow controlled expression of *MKKK7*, we used an inducible system that is based on the XVE chimeric transcription activator inducible by estradiol (Zuo et al., 2000).

Two independent non-segregating T3 transformant lines were selected for each construct and used for inoculations with virulent *Pst* DC3000. Sets of 4- to 5-week-old plants were sprayed with an estradiol solution in order to activate gene expression of the construct before inoculations. After dip inoculation the plants were scored at 3 dpi for disease symptoms. In Col-0 plants the percentage of leaves with disease symptoms was 51% (Fig. 9C). *Ind-MKKK7* showed a significant increase in disease symptoms. In *ind-MKKK7*^{AA} plants, expressing the non–phosphorylatable version of MKKK7, disease symptom development was comparable to that of Col-0. Overexpression of the phosphomimic version of *MKKK7* (*ind-MKKK7*^{DD}) resulted in a significant increase in disease susceptibility, albeit to a lesser extent than *ind-MKKK7* plants (Fig. 9C). Taken together these observations support the requirement of phosphorylation of MKKK7 at one or both Ser residues to effectively suppress basal immunity.

DISCUSSION

MKKK7 functions as a negative regulator of PTI through inhibition of MAPK activation

In this study we present evidence of the biological relevance of MKKK7 in modulating basal defense responses and basal immunity. Using a combined loss-of-function and gain-of-function approach we show that MKKK7 acts as a negative regulator of flg22-triggered signaling and defense gene expression and suppresses PTI. Relatively little is known about the involvement of MAPKKKs in mediating defense responses and only a few members of this family were studied in more detail. Our detailed analysis of the *mkkk7* mutant and *MKKK7* overexpressing lines shows that the flg22-triggered phosphorylation of at least three potential downstream MAP kinases, MPK3, MPK4 and MPK6, is affected by MKKK7 activity. These biochemical results are supported by experiments in mesophyll protoplasts, where induction with flg22 leads to the activation of Arabidopsis MPK3 and MPK6 upstream of *WKRY29* and *FRK1* expression. Flg22-induced MPK3 and MPK6 activation is required for expression of these genes, as overexpression of phosphothreonine lyase effector proteins HopAII or SpVC in mesophyll protoplasts completely blocks flg22-induced MAPK activation and downstream defense gene expression (Mithoe and Menke, data not shown)(Zhang et al., 2007). Flg22-stimulated *WRKY29* and *FRK1* expression can thus be used as a proxy for MAP kinase activation in Arabidopsis.

We observed that flg22-induced defense gene expression was effectively repressed when *MKKK7* or a phosphomimic version of *MKKK7* was co-transfected into protoplasts (Fig. 6B and Fig. S4), supporting the hypothesis that MKKK7 is a repressor of the flg22-induced MPK3 and MPK6 phosphorylation. Co-transfection of the non-phosphorylatable version of *MKKK7* did not block responsiveness to flg22. These results point towards a direct connection between phosphorylation of MKKK7 and its role as a suppressor of the flagellin responsive MAPK activation. Furthermore, since MPK3 and MPK6 are phosphorylated in

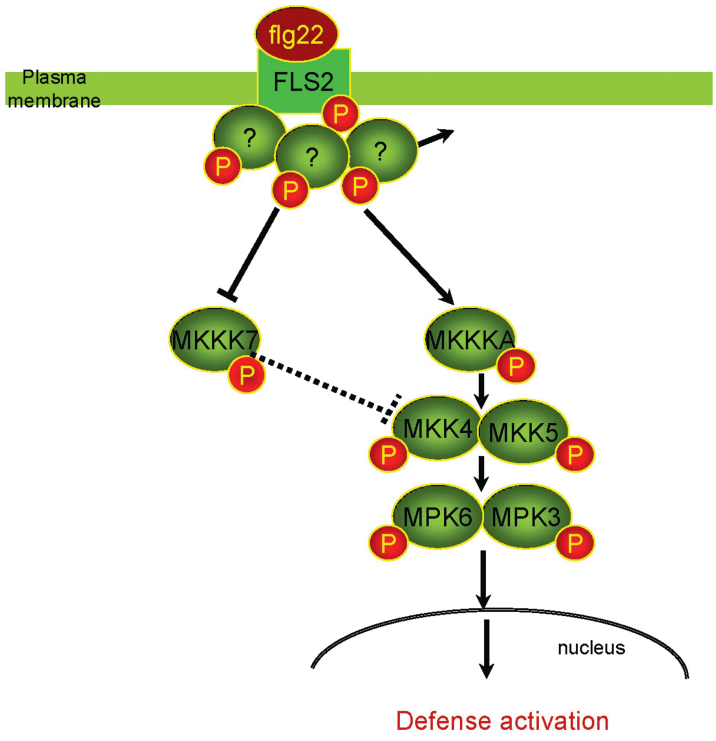

Figure 10. Model explaining MKKK7 function in PAMP-mediated signaling. FLS2 perceives the PAMP flg22 and that leads to the phosphorylation of unknown (?) signaling proteins and through unidentified intermediate steps leads to de phosphorylation and the activation of a 3-tiered MAPK module MKKKA-MKK4/5 and MPK3/6 and concomitant the dephosphorylation of MKKK7. Dephosphorylating MKKK7 releases inhibition of MPK3 and MPK6 phosphorylation and downstream defense responses.

response to flg22 within minutes, the connection between MKKK7 and MPK3 and MPK6 is likely regulated at the posttranslational level. This is also supported by available evidence for the role of MPK6 in basal immunity or PTI (Asai et al., 2002; Menke et al., 2004; Bartels et al., 2009; Anderson et al., 2011). MPK6-silenced lines displayed an enhanced susceptibility against avirulent and virulent strains of *P. syringae* (Menke et al., 2004) and a MEKK1-MKK4/MKK5-MPK3/MPK6 cascade was shown to be required for PTI against virulent bacterial and fungal pathogens (Asai et al., 2002). Furthermore, Arabidopsis MAP kinase phosphatase1 (MKP1), which targets MPK6, is observed as a negative regulator of PAMP responses and bacterial resistance (Bartels et al., 2009; Anderson et al., 2011). Similarly, loss-of-function mutant *mkkk7* displayed an increase in resistance against virulent *Pst* DC3000 while overexpression of *MKKK7* or *MKKK7*^{DD}, but not *MKKK7*^{AA}, resulted in enhanced susceptibility to virulent *Pst* DC3000. When all data is taken into consideration, it is highly likely that MKKK7 directly interacts with the MPK3/MPK6 cascade to negatively regulate flg22-induced

defense signaling and PTI (Fig. 10). It may well be that the function of MKKK7 is to repress basal signaling, through repression of steady-state levels of phosphorylated MAPKs in the absence of flg22 perception. Both *WKRY29* and *FRK1* had elevated mRNA levels in *mkkk7* compared to the wild-type Col-0, even prior to induction. As such, reduction of active MKKK7 protein, in the *mkkk7* mutant or through flg22-induced dephosphorylation could lead to a state of priming, in which the cells respond faster to PAMP perception. Priming of stress response has been shown to require MPK3 and MPK6 in Arabidopsis (Beckers et al., 2009) and as such the *mkkk7* mutant with slightly higher levels of MPK3 and MPK6 activity may actually be in a primed state of defense.

MKKK7 acts downstream of FLS2 and upstream of MPK3 and MPK6

To connect MKKK7 with the downstream MAPK cascade, yeast 2-hybrid (Y2H) experiments were carried out, but there were no specific interactions of MKKK7 with any MEK protein detected, while other MAPKKK family members were interacting with specific MEKs (data not shown). One of the possible causes of the failure to detect interactions could be low expression levels in yeast due to the size of the *MKKK7* gene and encoded protein, which is about 4.3 kb and 1368 amino acids in length. This made it difficult to connect MKKK7 to a specific MEK and downstream MAPK cascade. The identification of MPK3, MPK4 and MPK6 as a target of MKKK7 did also not really narrow down the possible interacting MEKs. MPK3 and MPK6 have been shown to act downstream of several different MEKs including MKK4 and MKK5 in response to flg22 perception (Asai et al., 2002) and MKK7 and MKK9 in ethylene signaling (Yoo et al., 2008), MKK3 in JA-response (Takahashi et al., 2007) and MKK1 in response to ABA signaling (Xing et al., 2008, 2009), implicating a total number of 6 MEKs in connection with MPK6 alone. It is well known from yeast and mammalian systems that individual MAPK cascade components can act in multiple signaling cascades, as exemplified by yeast Ste11, which functions in the pathway regulating cell wall synthesis and also in the pathway that regulates vegetative growth (Lee and Elion, 1999). In Arabidopsis there are only 10 MEKs as compared to 20 MAPKs and more than 80 MAPKKKs, therefore it is likely that MEKs function in more than one signaling pathway.

Negative regulation by MKKK7 of MPK6 activation could also happen through interaction with signaling components upstream of MEKs, including other MAPKKKs, or even components of the FLS2-BAK1 receptor complex. There is still no evidence how the perception of flg22 through the FLS2-BAK1 receptor complex is transferred to the downstream MAPKKK(s). Furthermore, which MAPKKKs may be regulating flg22 signaling in Arabidopsis upstream of the MKK4/MKK5-MPK3/MPK6 cascade is still a matter of debate (Asai et al., 2002; Suarez-Rodriguez et al., 2007). Several MAPKKK family members were identified in a proteomic screen as differentially phosphorylated in response to flg22, including two RAF-like kinases and MKKK3α (Menke et al, unpublished). Not much is known about these MAPKKKs in Arabidopsis, but the tobacco orthologue of MKKK3α is required for AvrPto mediated cell death and resistance against infection by *Pst* DC3000 (del Pozo

et al., 2004). This requires tobacco MEK2 and SIPK activity downstream of MKKKα, which are the tobacco orthologues of Arabidopsis MKK4/5 and MPK6 respectively. This would be in agreement with functional conservation of MKKK3α as a positive regulator of PTI in Arabidopsis, acting through a conserved cascade composed of the orthologues MKKs and MAPKs in Arabidopsis and tobacco. Since MKKK7 not only represses MPK3 and MPK6 phosphorylation and activity but also MPK4 activity, it is likely that MKKK7 is capable of interacting and inhibiting with several MKKKs or several MEKs that act in at least two MAPK cascades that are activated by FLS2 signaling.

MKKK7 is functionally different from MAPKKKεs

The tomato homologue of MKKK7 is SlMAPKKKε and was recently shown to be involved in the positive regulation of hypersensitive cell death and is also required for resistance against gram-negative bacterial pathogens in tomato (Melech-Bonfil and Sessa, 2010). Constitutive overexpression of Arabidopsis *MKKK7* resulted in a range of phenotypes including very small plants with altered leaf morphology, HR-like response and early senescence in the absence of pathogen inoculation. This could indicate that MKKK7 and SlMAPKKKε are functional orthologues.

However, contrary to tomato *MAPKKKε* overexpression, Arabidopsis plants over-expressing *MKKK7* were more susceptible to *Pst* DC3000 infection. Therefore, although MKKK7 is an orthologue of SlMAPKKKε based on homology at amino acid sequence level, the function in Arabidopsis does not appear to be similar and these kinases are therefore not functional orthologues.

Functional relevance of MKKK7 phosphorylation

Despite the functional relevance of phosphorylation of MAPKKKs, as was shown by STE20 mediated phosphorylation of MAPKKK STE11 in yeast (Drogen et al., 2000), only one plant MAPKKK involved in defense response has been described as a protein regulated by phosphorylation (Oh et al., 2010a). SlMAPKKKε abundance and activity are stabilized by phosphorylation on a C-terminal Ser residue and binding of the pSer residue by a 14-3-3 protein. The only other example of functional relevance of phosphorylation of a MAPKKK family member in plants is described in this chapter. We demonstrated that phosphorylation of MKKK7 is required for suppression of PAMP-triggered signaling via repression of MAPK phosphorylation resulting in reduced PTI. The two phosphorylated Ser residues identified here are in the central region of MKKK7 and flank an ARM domain. ARM domains are 40 amino acid repeats first identified in the Armadillo protein of *Drosophila* and mediate protein-protein interaction. The nature of the structure or sequence bound by an ARM repeat is in general not known and we can only speculate as to the role of the phosphorylation of the Ser residues flanking the ARM repeat. Since these phosphorylated residues are not in, or close to the activation loop of the kinase, these phosphorylation events are not likely to directly regulate the kinase activity of MKKK7. It is possible, that phosphorylation of MKKK7

is required for interaction with another protein that is part of a MAPK cascade inhibited by MKKK7.

We mutated both Ser residues that were differentially phosphorylated into either Ala or Asp. The choice to mutate both Ser sites in MKKK7 was based on equal ratios of phosphorylation of the Ser sites and the presence of a few similar amino acid residues surrounding these Ser residues. However we cannot exclude that only one of these residues is functionally relevant or that these residues may even have opposite regulatory function. There is precedence for this in ethylene signaling, where EIN3 is downstream of the CTR1 regulated MAPK cascade, which phosphorylates two threonine residues in EIN3. To determine the *in vivo* function of the phosphorylation sites in ethylene signaling, an EIN3 $^{T174A}/^{T592A}$ (EIN3^{AA}) mutant was generated. Analysis of the individual phosphorylation site mutants (EIN3T^{174A} or EIN3T^{592A}) lead to the unexpected discovery of opposite effects on EIN3 stability (Yoo et al., 2008; Yoo and Sheen, 2008). Analysis of single amino acid residue replacements would also be required to fully address this question for MKKK7 and constructs have been generated for this purpose.

Is MKKK7 function specific for FLS2 signaling?

It may well be that single amino acid substitution mutant versions of MKKK7 could shed light on some of the more complicated phenotypes, observed during analysis of the loss-of function and gain-of-function MKKK7 lines. One such phenotype is the inhibition of growth in response to flg22 and elf18. While flg22 modestly induced more inhibition of growth of the seedlings at lower concentrations of flg22, root growth in response to low concentrations of flg22 was not more sensitive, if anything it seemed less sensitive at the lowest concentration tested whereas elf18 inhibited root growth inhibition did not seem to be affected in the *mkkk7* mutant. Overexpression of *MKKK7* resulted in a complete insensitivity to flg22 when primary root growth was measured, while the same *ind-MKKK7* lines were still responsive to elf18. This latter result suggests that MKKK7 is more effective at suppressing flg22-mediated responses and may not regulate elf18 signaling.

Elf18 is perceived through EFR, a RLK that also requires BAK1 as a co-receptor. EFR seems to be confined to the *Brassicaceae* and is not found in other dicots or monocots, suggesting that EF-Tu perception is evolutionarily young (Boller and He, 2009). MKKK7 is conserved in *Brassicae* and other higher plants (Jouannic et al., 2001; Champion et al., 2004; Melech-Bonfil and Sessa, 2010). Flagellin responsiveness is shared by members of all higher plants, indicating that both FLS2 and MKKK7 dependent signaling is evolutionary ancient. While most PTI signaling events tested so far seem to be shared between FLS2 and EFR signaling cascades, there are so-called elfin mutants identified that specifically affect EFR mediated but not FLS2 mediated responses (Nekrasov et al., 2009; Nicaise et al., 2009; Schwessinger et al., 2011). Our current results show that MKKK7 is a functional component of the FLS2 signaling network and may not be a general inhibitor of PAMP signaling. Further experiments to distinguish between these possibilities are required.

Concluding remarks

In this chapter we describe the functional analysis of phospho-protein MKKK7, which was identified as differentially phosphorylated in response to flg22. MKKK7 negatively regulates PAMP-triggered signal transduction and PTI. MKKK7 acts by inhibiting MAPK phosphorylation and we show that this function requires phosphorylation of MKKK7 on specific Ser residues. Since MKKK7 is dephosphorylated on these Ser residues in response to flg22 perception, our data suggests that derepression of MKKK7-mediated inhibition of MAPK cascades is required for full activation of PTI signaling and PTI**.**

METHODS

Plant material and methods

All mutant and transgenic lines used in this study were in the background of *Arabidopsis thaliana* accession Columbia (Col-0). The loss-of function T-DNA insertion line *mkkk7* (SALK_133360) was generated by SIGnAL and obtained from the European Arabidopsis Stock Centre (NASC) in Nottingham, UK. Plants were grown on soil or on MS salt medium (Duchefa) with 1% sucrose and 1% agar. The mutant line *mkkk7* was backcrossed to Col-0 wild-type. All lines were grown under normal long-day growth conditions at 2°C and after 4 weeks leaf material was harvested and gDNA was isolated. The position of the insertion was confirmed by genotyping using PCR with gene-specific primers for *MKKK7* 5'-GCAGGATTTTTGTTGTTGTCC-3' and 5'-AATCATTTCTTGGGGTGGATC-3' and 5'-TGGTTCACGTAGTGGGCCATCG-3' for the left border of the T-DNA.

RNA extraction and qRT-PCR analyses

Material for RNA analysis was frozen in liquid nitrogen and stored at -80°C. For defense gene analysis duplicate sets of tissue was induced at set time points 0, 1, 2, 3, 4 h after induction with 10 µM of flg22. This experiment was duplicated with 8 µM of NAA to study auxin responsiveness with RT-PCR. Tissue was grounded in liquid nitrogen followed by extractions using TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA extractions for RT-PCR and qRT-PCR were performed as described in Menke et al. (2004). cDNA was synthesized from 1 µg of total RNA using SuperScript II reverse transcriptase (Invitrogen). All RT-PCR reactions were performed under the following conditions: 94°C for 3 min, 26 cycles (94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min), and a final extension at 72°C for 5 min. qRT-PCR was performed using the SYBR Green protocol (Applied Biosystems http://www.appliedbiosystems.com). Each marker gene was normalized to the internal reference gene *At2g29550 (TUB7*) and plotted relative to the Col-0 mock expression level. All primers designed for RT-PCR and qRT-PCR analyses of *MKKK7* are listed in Supplemental table S1.

SDS-PAGE and MAP kinase assay

Leaf material of 4 week old seedlings was cut into 0.5 cm thin strips and floated in a well with 1 ml of water of a 24 wells plate to recover from the wounding stress. After 20-24 h, time-course inductions were done with the synthetic peptide flg22 at t=0, 5, 10, 30 and 60 min. The material was frozen in liquid nitrogen and stored in -80°C. Protein extractions and SDS-PAGE were performed as described in Menke et al. (2004). Equal loading was checked by Ponceau S staining and membranes were rinsed in TBS with Tween20 (TTBS), blocked for 1 h in TTBS with 5% nonfat milk powder and incubated overnight at 4°C with polyclonal primary rabbit antibodies raised against MPK3 (a-C-3, 7.5 µg/mL) or MPK6 (a-N-6, 5 µg/mL) diluted in TTBS solution with 3% BSA (Sigma). Membranes were rinsed 4 times in TTBS before incubation with the secondary antibody anti-rabbit-HRP conjugated (1:2000, Cell Signaling). As a loading control membranes were incubated with α-Actin Mouse IgG, clone C4 antibody (1:1000, ICN), followed by incubation with the secondary antibody anti-mouse-HRP conjugated (1:5000, Novagen). MAP kinase activity was detected using anti-phospho-p42/44 MAPK (Thr202/Tyr204) primary antibody (1:750, Cell Signaling, Bioke) in TTBS with 3% BSA at 4°C for 16-20 h. Blots were washed as described above after which incubation was continued

with a 2 h incubation with anti-rabbit-HRP conjugated secondary antibody (1:2500, Cell Signaling). Antigen-antibody complexes were visualized using chemiluminescence detection with ECL Western Blotting Detection Kit (GE Healthcare) according to the manufacturer's instructions before exposure to film (Kodak).

PAMP-induced growth inhibition

PAMP-induced growth inhibition was performed as described (Bardoel et al., 2011). Harvested seeds of Col-0, mutant *mkkk7*, transgenic *ind-MKKK7* lines, and the *fls2* mutant were sterilized and stratified for 2 days at 4°C in the dark. Seeds were sown in liquid MS media for fresh weight measurement and transferred into the light. Five-day-old seedlings were transferred into liquid MS with or without the indicated amount of flg22 and incubated for another eight days. Dry weight of 3 replicates per treatment was measured using a precision scale (Sartorius) and plotted relative to untreated control. For PAMP-induced primary root growth analyses, seeds were shown on agar solidified ½ MS medium and grown for 5 days, after which they were transferred to solid ½ MS medium with 2 µM estradiol and varying concentrations of the PAMPs flg22 or elf18. Primary root growth was registered every 24 h by marking the position of the root tip. Primary root length was measured using Fiji software (Image J) on calibrated photo's at 9 days post transfer.

Mesophyll protoplast assay

To study transient gene expression, Arabidopsis plants were grown in short-day growth conditions. Mesophyll protoplast isolation and transfections of plasmid DNA was conducted as described (Yoo et al., 2007). To study early transcription responses, three plasmids expressing a regulatory effector, a specific reporter and a transfection control reporter were transfected at the ratio of 4:3:1. Ten µM of the synthetic peptide flg22 or 8 µM of NAA was added after 16h incubation of protoplasts at 22°C. The relative fLUC reporter activity of the defense-or auxin responsive genes was measured against the rLUC activity using the Dual Luciferase reporter assay system kit (Promega, Madison, USA) according to the manufacturer's instructions. The LUC activity was measured using the TD-20/20 Glomax luminometer (Promega). All fLUC activity was normalized to the non-treated wild type**.** Constructs used to test PTI in protoplasts are listed in Supplemental table S2.

Generation of transgenic plants

Different promoters were used to study the expression of the *MKKK7* gene. The MultiSite Gateway manufacturer's protocol was used to design primers to clone different promoters in BOX1 entry clone. The *35S* promoter, *pG1090::XVE* and *pMKKK7* were cloned in BOX1. *pG1090::XVE* is an estrogen receptor-based chemical inducible system (Zuo et al., 2000) to generate transgenic plants. The second entry clone BOX2 consisted either of the gDNA or the cDNA sequence of *MKKK7*. The point mutations of the Ser sites, which were observed in the large-scale phosphoproteomics study carried out by Benschop et al. (2007) was done according to the manufacturer's instructions for the Stratagene quick change mutagenesis kit. Ser (452) and Ser (854) were both changed to Ala, a non-phosphorylatable version and to Asp, a phosphomimic version. BOX3 of the gateway system either had the marker GFP or NOS terminator. The integrity and sequence of all entry clones was confirmed by sequencing. The correct entry clones were combined to one construct (LR reaction). This final constructs were checked by restriction digestion. Primers used for PCR amplification for the MultiSite Gateway cloning and for the quick change mutagenesis are listed in Supplemental table S1. Transgenic plants were generated by electroporation of the constructs into *Agrobacterium tumefaciens* strain C58. All constructs were transformed into Arabidopsis mutant *mkkk7* and Col-0 using the floral dipping method. Transformants were selected on ½ MS agar medium containing 40 µg/ml Norf, *35S::MKKK7:GFP* and amino acid substituted derivative overexpressor constructs were used in the mesophyll protoplast system to study gene transcription. All generated constructs are listed in Supplemental Table S3.

Pathogen inoculation and analysis of resistance

Plants were individually transplanted into soil and grown for 4 weeks in shortday conditions. Prior to pathogen inoculation, *P. syringae* pv.tomato DC3000 (*Pst* DC3000) was grown in overnight culture in Kings B medium supplemented with appropriate antibiotics as described (Menke et al., 2004). Cells were harvested by centrifugation and pellets re-suspended in sterile 10 mM $MgSO_4$ to OD600 = 0.2. Silwet L-77 (0.015%) was added to the bacterial

suspension to a concentration of 0.04% (v/v) prior to dipping. Inducible transgenic lines carrying *MKKK7*, *MKKK7*^{AA} and *MKKK7*^{DD} were sprayed with dexamethasone solution 24 h prior to inoculation. Plants were dipped into the bacterial suspension and returned to the short-day growth chambers. After 2 days, the disease symptoms were observed every day for 3 days. To quantify the disease symptoms after pathogen infection, samples were taken using a cork-borer (2 mm) to cut leaf discs from 2 leaves per plant and 3 plants per genotype. Leaf disks were ground in 10 mM $MgCl_2$, and dilutions were plated in Kings B medium with 40 mg/mL of rifampicin and 50 mg/mL of kanamycin and incubated at 27°C for 48 h, after which the number of colonies formed were counted. Each dilution was plated in triplicate. At least 2 biological replicates were done.

ACKNOWLEDGEMENTS

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SUPPLEMENTAL DATA

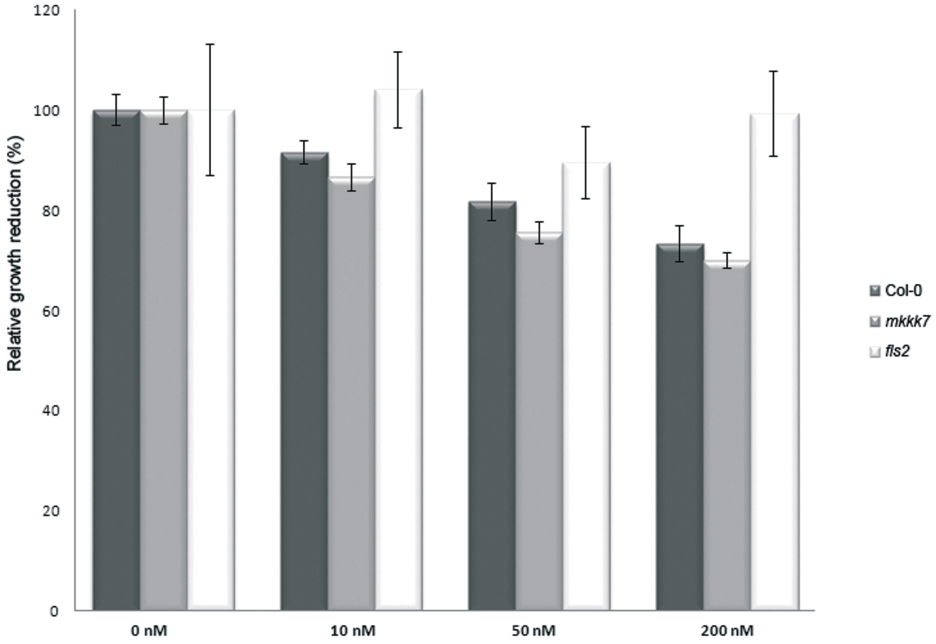

Figure S1. *mkkk7* mutants show a modest sensitivity to flg22 in seedling growth. Seedling growth assay of 10 seedlings of Col-0, *mkkk7* and *fls2* after treatment with flg22. The fresh weight of the seedlings was measured 8 days after transfer to ½ MS medium with 0, 10, 50 and 200 nM flg22. The results are shown as fresh weight of the flg22-treated seedlings relative to that of the mock treatment. Data represents the mean values ± SEM (*n*=3). Experiments were repeated 6 times with similar results.

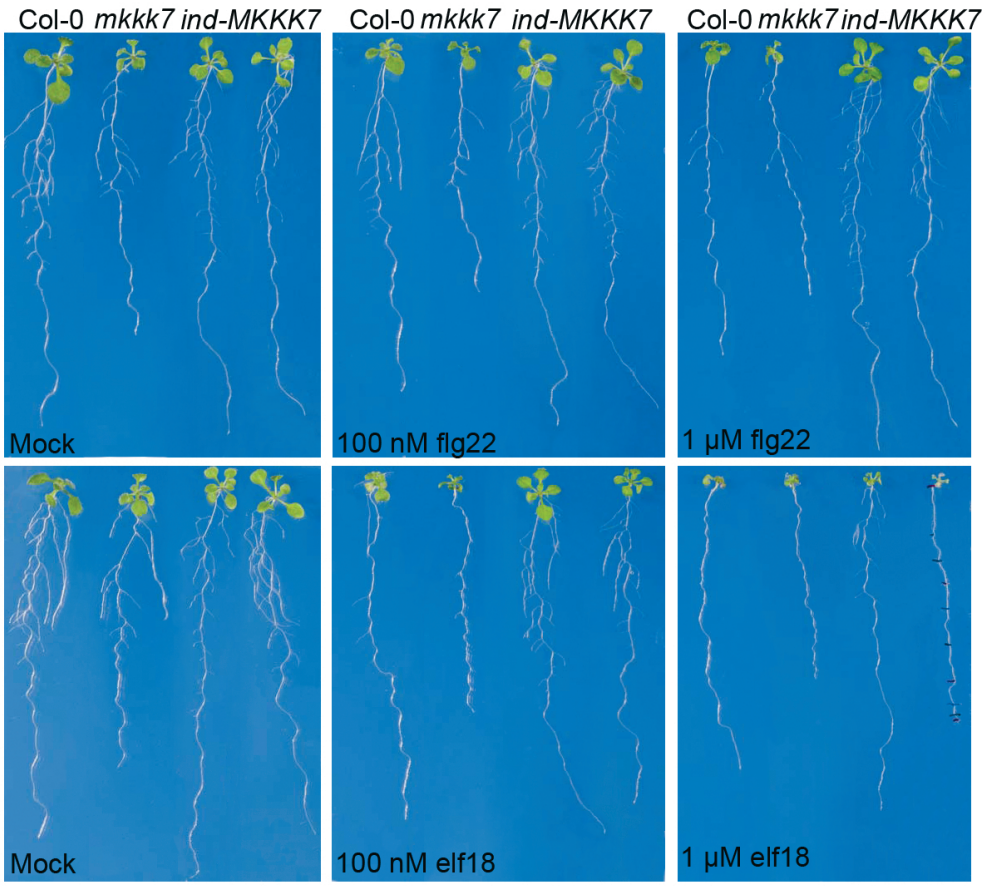

Figure S2. *MKKK7* overexpression leads to seedlings insensitivity of flg22-induced growth inhibition. Dose-dependent inhibition of seedling and primary root growth by the PAMPs flg22 and elf18. Five-day-old seedlings were transferred to agar solidified ½ MS medium with 2 µM estradiol and indicated concentrations of flg22 or elf18 and growth was recorded 8 to 9 days later. One representative seedling out of 10 seedlings is shown for each genotype and each treatment. Genotypes shown at the top panel: Col-0, *mkkk7* mutant and two independent estradiol inducible *MKKK7:GFP* (*ind-MKKK7*) lines. Treatment is shown below the roots. Experiment was done twice with similar outcome.

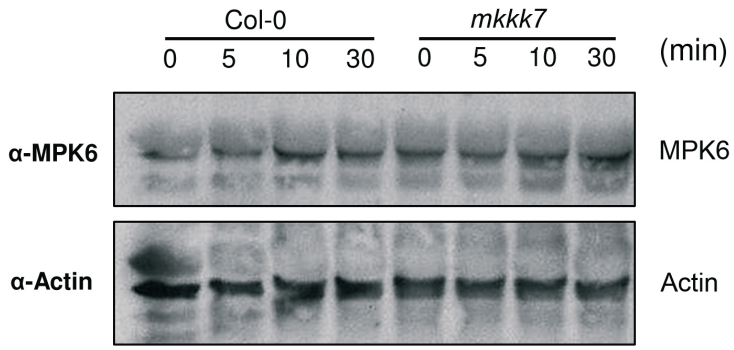

Figure S3. **MPK6 protein levels are unaltered in the *mkkk7* mutant**. Leaf tissue from 4-week-old plants were treated or not with 1 µM flg22 and samples were taken at t=0, 5, 10, 30 min. Immunoblot was incubated with α-MPK6 antibody to detect MPK6 protein levels (top panel) and α-Actin antibody was used as a loading control (bottom panel).

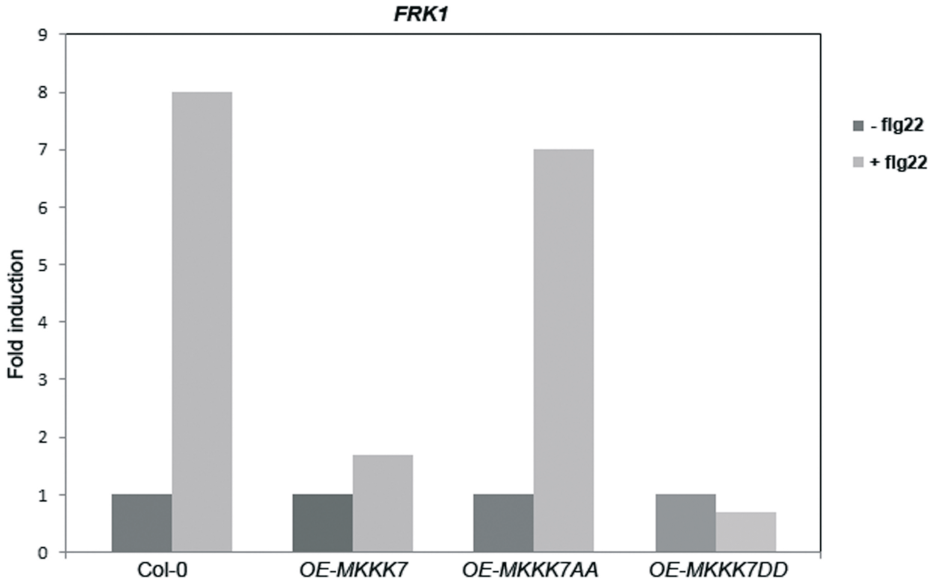

Figure S4. MKKK7 modulates flg22-induced *FRK1* gene expression. Transient co-expression of *MKKK7* in Arabidopsis mesophyll protoplasts shows suppression of *FRK1* gene expression in protoplasts after flg22 treatment. Protoplasts were isolated from 4-week-old plants and transfected with *pFRK1::fLUC, 35S::rLUC* and overexpression construct of *MKKK7* (*OE-MKKK7, OE-MKKK7^{AA} or OE-MKKK7^{DD}*) as indicated at the right of the graph. Sixteen hours later, protoplasts were treated with 10 µM flg22. All measurements were normalized to the rLUC activity and expression levels were calculated relative to the mock treated control sample as shown as fold induction.

Table S1: Primers designed for RT-PCR, qRT-PCR and cloning of MKKK7 cDNA.

Method	Primer name	Sequence 5'-3'
RT-PCR	*MKKK7* F1	GCACGTTGGCAAAGAATTATCC
	MKKK7 R1	GCAGCTATACGGCAGAAATCA
	Actin2 FW	GCACCCTGTTCTTCTTACCG
	Actin RV	AACCCTCGTAGATTGGCACA
	ATFRK1 FW	TAGATGCAGCGCAAGGACTA
	ATFRK1 RV	CTTGCTCGAGGAACCATCTC
	GH3 FW	AACCCGTCTCTCTTGCTTCTCA
	GH3 RV	CAACGTTCACACCAATCCAG
QRT-PCR	FM220	GAAATCATATCGGATCAATAAGACA
QRT-PCR	FM221	GGCCGTGGATGATGTTCATA
QRT-PCR	FM208	GGAAGAAGCTGAGTACGAGCA
QRT-PCR	FM209	GCAACTGGAAGTTGAGGTGTT
Cloning of *MKKK7* in gateway boxII	BoxII *MKKK7* FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGGCGCGGCAA ATGACG
	BoxII *MKKK7* fusion REV	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAATATTGTGTTGAT GTGAAGTGC
	1354bp Ser to Ala mut1 *MKKK7* compl	CGAAAGGCTGTGAAGACTCCAGCTAGTGTTAGTGGGAATGAAC
	1354bp Ser to Ala mut1 *MKKK7* coding	GTTCATTCCCACTAACACTAGCTGGAGTCTTCACAGCCTTTCG
	1354bp Ser to Asp mut1 *MKKK7* compl	CGAAAGGCTGTGAAGACTCCAGATAGTGTTAGTGGGAATGAAC
	13564bp Ser to Asp mut1 *MKKK7* coding	GTTCATTCCCACTAACACTTTCTGGAGTCTTCACAGCCTTTCG
	2560bp Ser to Ala mut2 *MKKK7* compl	GGTGATAAGCCTAGAGTAGCCAGTGTTGCACCAGATGC
	2560bp Ser to Ala mut2 *MKKK7* coding	GCATCTGGTGCAACACTCGCTACTCTAGGCTTATCACC
	2560bp Ser to Asp mut2 *MKKK7* compl	GGTGATAAGCCTAGAGTAGACAGTGTTGCACCAGA
	2560bp Ser to Asp mut2 *MKKK7* coding	GCTGGTGCAACACTCTCTACTCTAGGCTTATCACC

Table S2: Constructs for testing PAMP-triggered immunity responses in protoplasts.

Purpose	ARBC stock number	Reporter	Promotor
Transfection control	CD3-911	*pHBT-sGFP(S65T)-NOS*	35S derivative
Specific reporter	CD3-914	*pWRKY29-LUC-NOS*	At4g23550
	CD3-919	*pFRK1-LUC-NOS*	At2g19190
	CD3-913	*pAtGH3-LUC-NOS*	At2g23710
Regulatory gene		*MKKK7-gfp6hisnosT*	35S
		MKKK7^{AA}-gfp6hisnosT	35S
		MKKK7^{DD}-gfp6hisnosT	35S

Table S3: *MKKK7* constructs for transfection in protoplasts and complementation in plants.

Purpose	Construct	Abbreviation
Defense response in protoplasts	*35S::MKKK7:GFP*	*OE-MKKK7*
	35S::MKKK7 $^{SS->AA}$:GFP	*OE-MKKK7^{AA}*
	35S::MKKK7$^{SS->DD}$:GFP	*OE-MKKK7^{DD}*
Complementation in plants	*35S::MKKK7:GFP*	*OE-MKKK7*
	35S::MKKK7:NOST	Not described
	pMKKK7::MKKK7:GFP	Not abbreviated
	pMKKK7::MKKK7:NOST	Not described
	pG1090::XVE: UAS::MKKK7:GFP	*ind-MKKK7*
	pG1090::XVE: UAS::MKKK7$^{SS->AA}$:GFP	*ind-MKKK7^{AA}*
	pG1090::XVE UAS:MKKK7 $^{SS->DD}$:GFP	*ind-MKKK7^{DD}*

CHAPTER 5

5

MKKK7 is involved in maintenance of Arabidopsis root meristem size and root growth

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ABSTRACT

Root meristem maintenance is a model system to study postembryonic development in plants. Significant progress has been made in elucidating the signaling pathways involved in root development. Based on genetic evidence we report that *mitogen-activated protein kinase kinase kinase 7* (*MKKK7)* is involved in root meristem maintenance and meristem cell proliferation, which are required for root growth. Root growth of the loss-of function *mkkk7* mutant was impaired and corresponded with a reduction of the number of cells in the root meristem and elongation zone. Overexpressing the wild type *MKKK7* resulted in a significant increase in cell number in the root meristem, whereas point mutations affecting the phosphorylation status of MKKK7 significantly reduced this increase. The *mkkk7* short-root phenotype can be rescued with the addition of tyrosine sulfated peptides of the root meristem growth factors (RGFs)/Golven (GLV) family involved in meristem maintenance and meristem cell proliferation. Our results suggest that the phospho-protein MKKK7 acts upstream of peptide-mediated cell-cell signaling to control root meristem size and meristem cell proliferation.

INTRODUCTION

Plants are known for their ability to modulate their endogenous developmental programs in order to survive unfavorable conditions. This developmental plasticity enables plants to outgrow competitors, reach sources of nutrients and water in their immediate surroundings and modulate growth while fending off microbial pathogens (Achard et al., 2003; Malamy, 2005; Navarro et al., 2006). Developmental plasticity in roots allows flexibility in regulation of root growth, as well as root architecture. Due to its simplicity of organization, roots are an excellent model to study developmental programs, including the mechanisms that regulate developmental plasticity.

Root growth is determined by the activity of the root apical meristem (RAM) at the tip of the growing root. The meristem is maintained by the organizing center or quiescent center (QC) and its surrounding stem cells (Stahl and Simon, 2009; Bennett and Scheres, 2010). The activity of the stem cell niche located in the root tip is required for meristem maintenance and root growth. Several transcription factors required for QC specification and maintenance have been identified and act in two parallel pathways, which are essential for activity of the QC: the PLETHORA (PLT) pathway (Aida et al., 2004; Galinha et al., 2007) and the SHORT-ROOT (SHR)/ SCARECROW (SCR)/RETINOBLASTOMA RELATED (RBR) pathway (Helariutta et al., 2000; Nakajima et al., 2001; Sabatini et al., 2003; Wildwater et al., 2005). PLT1 and PLT2 proteins act as dose-dependent regulators of root development, possibly acting upon auxin gradients in the meristem (Galinha et al., 2007). The stem cells generate self-renewing daughter cells and transient amplifying daughter cells, which continue to divide, gradually moving further away from the QC area through the proximal meristem, until they reach the transition zone where they start to elongate and differentiate finally reaching maturity in the differentiation zone (Vanstraelen and Benková, 2012). *PLT1* and *PLT2* genes are transcribed in the RAM in response to the phytohormone auxin and they require auxin response transcription factors (ARFs) to form gradients that are thought to be a readout of an underlying auxin gradient (Aida et al., 2004; Galinha et al., 2007; Grieneisen et al., 2007). Additional pathways that regulate the *PLT* genes have hitherto not been identified.

Crosstalk in the feedback mechanisms between auxin and cytokinin is needed for establishment and maintenance of the RAM (Stahl and Simon, 2009; Perilli et al., 2010). Higher levels of auxin in the root meristem are generally associated with cell division, while higher levels of cytokinins are associated with differentiation. The balance between auxin and cytokinin signaling determines the position of the transition zone, where cells transit from meristematic divisions to enter into elongation and differentiation (Dello Ioio et al., 2008). The relative balance of auxin and cytokinin together thus determines root meristem size.

In addition to the aforementioned phytohormones auxin and cytokinin, peptides mediating cell-cell signaling are also involved in meristem maintenance, cellular proliferation and elongation (Katsir et al., 2011). A subset of these peptides, called phytosulfokines, significantly promote cellular proliferation and expansion (Matsubayashi and Sakagami,

1996; Amano et al., 2007; Matsuzaki et al., 2010). The most striking feature of these peptides is their posttranslational modification through sulfation on tyrosine residues, which occurs in both animals and plants. Tyrosine sulfation is mediated by tyrosylprotein sulfotransferase (TPST), which catalyzes the transfer of sulfate to the phenolic group of tyrosine residues (Yu et al., 2007). A recently identified family of secreted tyrosine-sulfated peptides called root meristem growth factors (RGFs), was reported to play a critical role in root meristem maintenance (Matsuzaki et al., 2010). Triple *rgf1 rgf2 rgf3* mutants have reduced root meristem size, and the short-root phenotype was restored by the external application of RGF1 peptide. RGFs are required for maintenance of the root stem cell niche and transit amplifying cell proliferation in *Arabidopsis thaliana* (Arabidopsis). The downstream transcriptional targets include *PLT1* and *PLT2* (Matsuzaki et al., 2010) providing a first link between cell-cell signaling and auxin response. A subset of the *RGF* gene family was identified as GOLVEN (GLV) peptides, which directly modulate the distribution of the phytohormone auxin, via modulation of auxin efflux carrier PIN proteins (Whitford et al., 2012).

Mitogen-activated protein kinases (MAPKs) play a central role in signaling both intrinsic as well as environmental cues in all eukaryotic cell types. Plant MAPK cascades have been implicated in a diverse array of biotic and abiotic stress responses as well as regulation of developmental programs (Pitzschke et al., 2009; Rodriguez et al., 2010). The best characterized of these developmental programs regulated by MAPKs is stomata development. Stomatal patterning involves the peptide ligand EPIDERMAL PATTERNING FACTOR 1 (EPF1) and receptor like kinases TOO MANY MOUTHS (TMM) and ERECTA-likes upstream of activation of the YODA, MEK4/5, MPK3/6 cascade (Dong et al., 2010). This MAPK cascade negatively regulates the transcription factor SPEECHLESS (Lampard et al., 2008) required for initial division of the meristemoid mother cell (MacAlister et al., 2007). This shows that meristematic divisions in leaf epidermal cells are controlled by a functional MAP kinase.

Kovtun et al. (1998) showed that activation of a MAPK cascade leads to suppression of early auxin-response gene transcription. However, a more extensive body of work has linked the same tobacco nucleus- and phragmoplast-localized protein kinase 1 (NPK1) and *Arabidopsis* ANP1 related MKKKs, and the downstream MEK6 and MPK4 kinases, to cytokinesis (Nishihama et al., 2001; Soyano et al., 2003; Takahashi et al., 2004; Sasabe et al., 2006; Beck et al., 2010; Sasabe et al., 2011).

Here we show that Arabidopsis MKKK7 (AtMAP3Kε1) functions as a positive regulator of root meristem maintenance. The *mkkk7* loss-of function mutant displays a short-root phenotype and a reduced root proximal meristem. Overexpressing *MKKK7* enhances root meristem size and root length, which we link to its phosphorylation status. Application of exogenous RGF/GLV peptides can rescue the meristem size phenotype of *mkkk*7 suggesting that MKKK7 acts upstream or in parallel to the peptide signaling pathway to control proximal root meristem size and meristem cell proliferation.

RESULTS

Knocking down *MKKK7* affects root growth and meristem size

Out of our phosphoproteomics screen for proteins phosphorylated in response to perception of pathogen-associated molecular patterns (PAMPs) (Benschop et al., 2007), we identified a number of candidates for functional analysis. We obtained T-DNA insertion lines for 5 candidate genes and verified the T-DNA insertion into the genes of interest. *MKKK7* (*At3g13530*) encodes a member of the MAPKKK family and belongs to the subfamily of the MEKKs (subgroup A4) (Ichimura et al., 2002). MKKK7 was identified as a negative regulator of PAMP-triggered signaling and PAMP- triggered immunity (PTI) in chapter 4, this thesis. In addition the homozygous knock-down mutant *mkkk7* showed a root growth phenotype that clearly manifested from about 5 days post germination (dpg) (Fig. 1A and 1B). Quantification

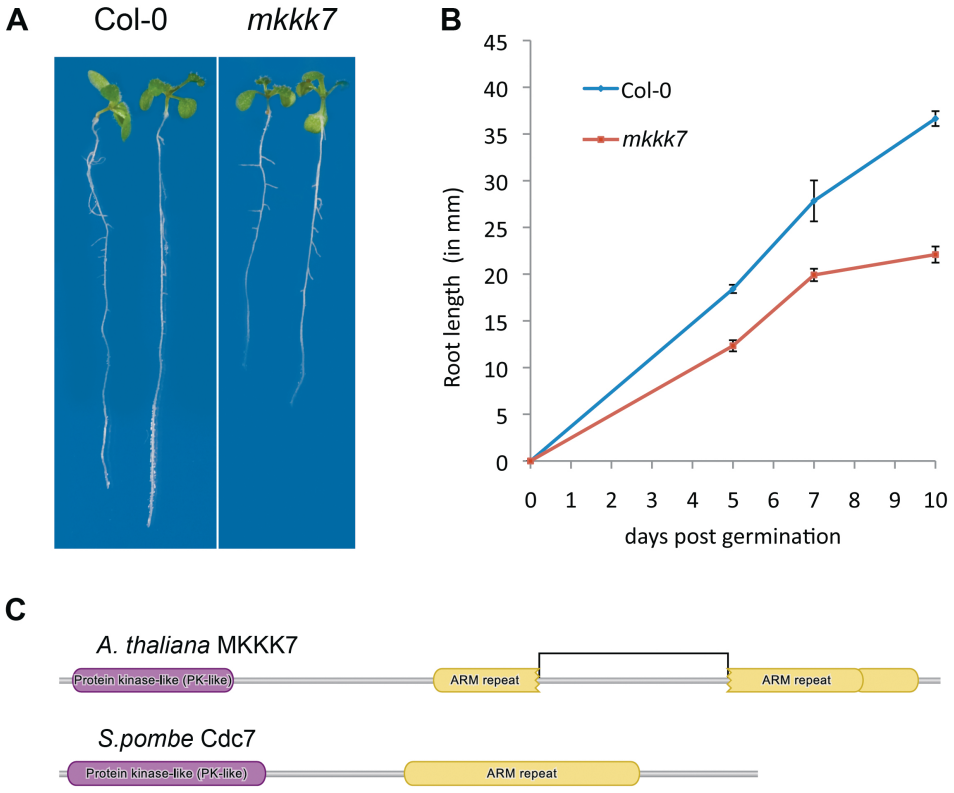

Figure 1. *Arabidopsis* root phenotype of wild type Col-0 and *mkkk7*. A) Comparison of the root length of Col-0 and *mkkk7* seedlings grown on vertical half-strength MS agar plates 7 days post germination (dpg). **B)** Quantification of the root length of Col-0 and *mkkk7* (*n*=10; error bars ± SEM.) **C)** Comparison of protein structure of MKKK7 with the orthologue Cdc7 from *Schizosaccharomyces pombe*. The kinase domain is shown in purple while ARM repeat domains are shown in yellow.

of the length of the primary root between 5 and 10 dpg showed that root length and the root growth rate were reduced compared to the wild type Col-0 (Fig. 1B). We also analyzed the germination rate of *mkkk7* to verify whether the shorter root phenotype could be partially due to a delay in germination. Under normal long day growth conditions *mkkk7* didn't display a delayed germination phenotype as compared to wild type Col-0 and at 3 dpg development of root and cotyledons appear normal (data not shown). Thus *mkkk7* has a short-root phenotype.

To understand the function of *MKKK7* we performed a phylogenetic analysis to identify orthologues. Highly conserved orthologues could be identified in all tested plant species, but also in more distantly related fungal species (Fig. S1). One of the best characterized orthologues is Cdc*7* from the fission yeast *Schizosaccharomyces pombe* (*S. pombe*), which is involved in signaling via the septation initiation network (SIN) pathway required to link mitosis and cytokinesis (Bedhomme et al., 2008). Sequence alignment and HMM (Hidden Markov Models) domain scans of MKKK7 and *S. pombe* Cdc7 revealed a high conservation of the protein kinase domain as well as the C-terminal part of the protein, which included an armadillo repeat domain (ARM) (Fig. 1C and S1). This computational analysis may indicate a role for MKKK7 in a cell cycle-related signal transduction pathway.

Based on the short-root phenotype and bioinformatic analysis, a function in controlling cell division for *MKKK7* was investigated by analyzing the root meristem size and organization using confocal microscopy. No obvious differences in the meristem organization could be observed and radial patterning as well as the QC area appeared normal as compared to Col-0 (Fig. 2A). The shorter distance between the QC area and the differentiation zone in the *mkkk7* mutant was the most obvious phenotype (Fig. 2A). Detailed analysis of the primary roots revealed that the short-root phenotype was the result of a reduction of both root meristem and elongation zone, compared to wild type (Fig. 2B). The meristem phenotype was mild, but quantification of the meristem size by counting the number of meristematic cortex cells revealed that the cell number in the proximal meristem of *mkkk7* was significantly reduced (Fig. 2B).

Furthermore, quantification of the number of cells in the elongation zone showed a more dramatic reduction in cells in the elongation zone in the mutant (*n*=10, 4 cells) compared to wild type (*n*=10, 6-7 cells) (Fig. 2C). The cell size in the meristem, elongation zone and differentiation zone were not observed to be different (Fig. 2A). Our data shows that knocking down *MKKK7* results in a shorter root phenotype and this is likely caused by a reduction in the number of cells in the root proximal meristem and elongation zone.

Restoring expression of *MKKK7* rescues the mutant phenotype

Since T-DNA insertion lines were shown to have multiple independent T-DNA insertions we investigated whether restoring *MKKK7* expression levels was able to rescue the mutant phenotype. A *pMKKK7::MKKK7*:*GFP* construct was introduced in the *mkkk7* mutant by *Agrobacterium tumefaciens*-mediated transformation and resistant T2 seedlings of two independent lines were screened for root length. *Expressing MKKK7:GFP* resulted in a root

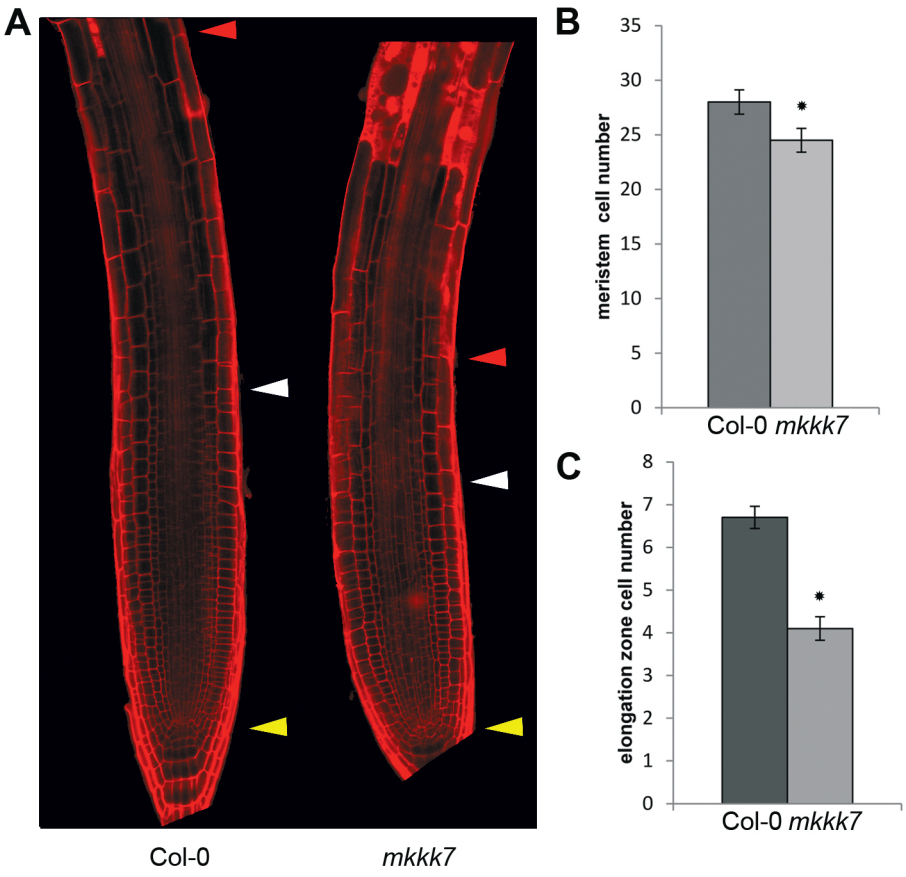

Figure 2. Characterization of *mkkk7* root meristem. **A)** Confocal images of the root tip of propidium iodide stained roots 5 dpg, the root meristem extends from the quiescent center (QC, yellow arrowheads) to the first elongated cell in the in the transition-elongation zone (white arrowheads). Red arrowheads indicate the position of cells showing first signs of differentiation. **B)** Quantification of the number of meristematic cortex cells of Col-0 and *mkkk7*. Data represents mean values ± SEM (*n*=10; *, P<0.05; paired t-test). **C)** Quantification of the number of cells in the elongation zone of Col-0 and *mkkk7*. Data represents mean values ± SEM (*n*=10; *, P<0.05; paired t-test). Experiments were repeated twice with similar results.

length of the transgenic seedlings which was comparable to Col-0 (Fig. 3A). Confocal analysis of the roots of the transgenic seedlings expressing *MKKK7:GFP* indicated that the increase in root growth was correlated to a recovery of the number of cortex cells in the root meristem to wild type levels (Fig. 3B). This shows that the *MKKK7:GFP* fusion gene product under the control of its own promoter fully complemented the root and meristem phenotype. Taken together these results suggest that *MKKK7* plays a regulatory role in maintenance of the root meristem size.

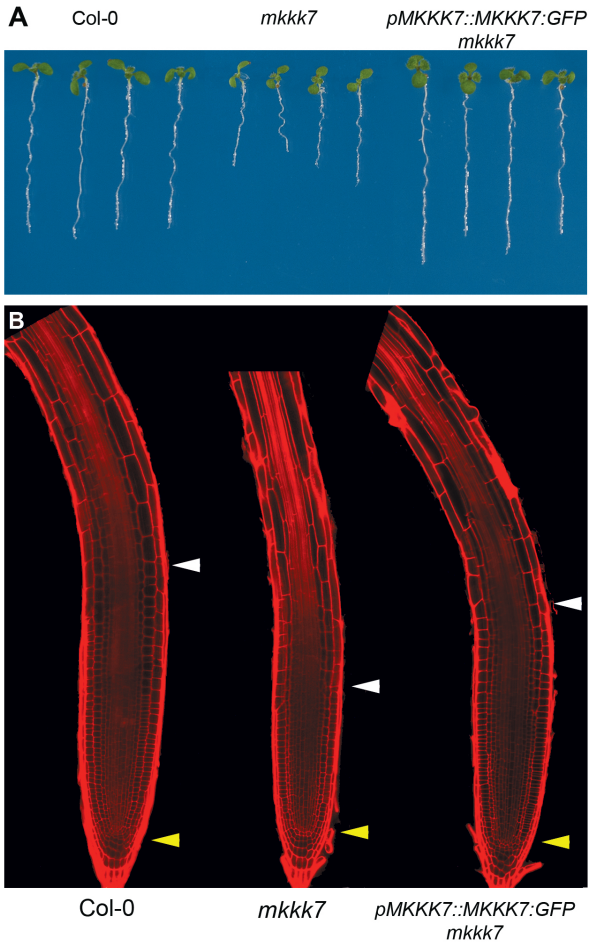

Figure 3. Complementation of *mkkk7* with a *MKKK7:GFP* fusion. A) Root length of 5 dpg old seedlings of Col-0, *mkkk7* and *mkkk7* complemented with *pMKKK7::MKKK7:GFP*. **B)** Confocal images of root tips of 5 dpg old seedlings of Col-0, *mkkk7* and *mkkk7* complemented with *pMKKK7::MKKK7:GFP*. The meristem size is indicated between the arrowheads (yellow arrowheads indicating the QC area; white arrowheads indicating the proximal meristem boundary).

Overexpression of *MKKK7* induces wavy root growth and an enlarged root meristem

To investigate whether overexpression of *MKKK7* affected root growth and meristem size, the *35S::MKKK7:GFP* (*OE-MKKK7*) construct was expressed in the *mkkk7* background. Based on resistance to selection marker Norf, we selected seedlings of two T2 transgenic lines and verified *MKKK7* gene expression in overexpression lines by qRT-PCR analysis (Fig. 7E, chapter 4 this thesis). Neither of these lines displayed an obvious increase in root growth but the lines showed a marked increase in root hair length (Fig. 4A).

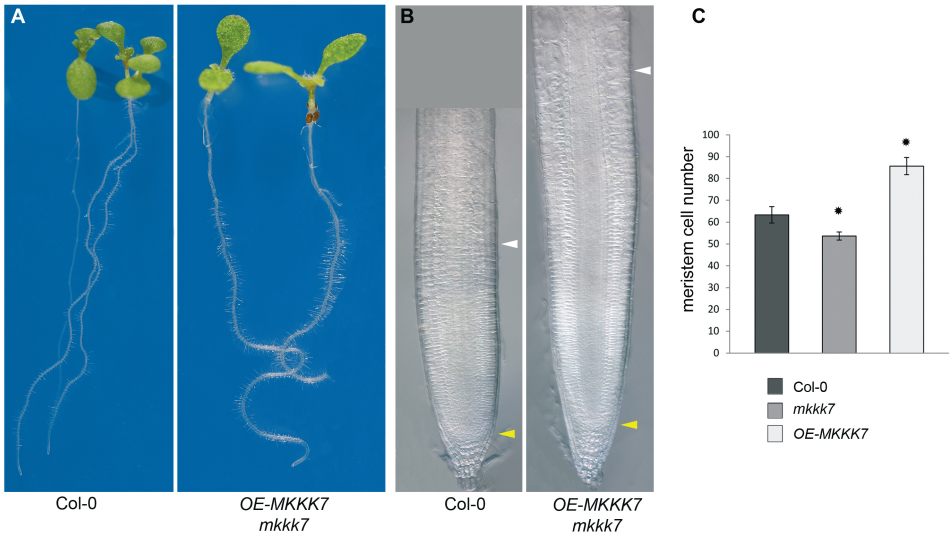

Figure 4. *MKKK7* overexpression phenotypes. A) Seedlings of one of two *OE-MKKK7* lines (right panel) at 7 dpg showing a wavy root and long root hair phenotype as compared to Col-0 (left panel). **B)** Nomarski images of the root meristem of *OE-MKKK7* transgenic line (right panel) compared to Col-0 (left panel) at 9 dpg. Yellow arrowheads indicate the QC area, white arrowheads indicate the upper boundary of the root meristem. **C)** Quantification of the number of meristematic cortex cells in the root of Col-0, *mkkk7*, *OE-MKKK7* at 9 dpg. Data represents mean values ± SEM (*n*=3; *, P<0.05; paired t-test).

In addition we observed severe bending of the growing root in one of these lines, suggesting a possible altered root gravitropic response (Fig. 4A). Detailed observation of the meristem of *OE-MKKK7* showed a large increase in meristem size without apparent increase in cell size. When we counted the number of cortex cells in the meristematic zone of *OE-MKKK7* it showed significantly more cells as compared to Col-0 (Fig. 4B and 4C). In addition we observed in the *OE-MKKK7* line a change in cell shape in the area of the presumed elongation zone. Instead of cell elongation, a more isodiametric cell expansion was observed, leading to a more conic shape of the root tip (Fig. 4B). This latter observation is also consistent with the absence of a longer root phenotype in the *OE-MKKK7* line and may be linked to enhanced bending of the root. Thus, *MKKK7* overexpression results in bending of the root, enhanced root hair length and a significant increase of the root meristem size.

Phosphorylation of MKKK7 is required to enhance root meristem size

To study the effect of controlled overexpression of *MKKK7* on root development, we generated estradiol inducible constructs. Out of 6 transgenic T2 lines we selected 2 lines with clear inducible expression of the MKKK7:GFP fusion protein (*ind-MKKK7*) (Fig. S2, upper panel). The T3 non-segregating progeny was analyzed for enhanced root growth and

changes in meristem size. Seeds were germinated under non-inducing conditions and at 2 dpg seedlings were transferred to medium containing low levels of estradiol and left to grow for 7 days. The root growth was quantified for 2 independent transgenic lines and revealed that controlled expression of *MKKK7* leads to a significant increase of the root length as compared to Col-0 (Fig. 5A and 5B). We did not observe enhanced bending or waving of the roots.

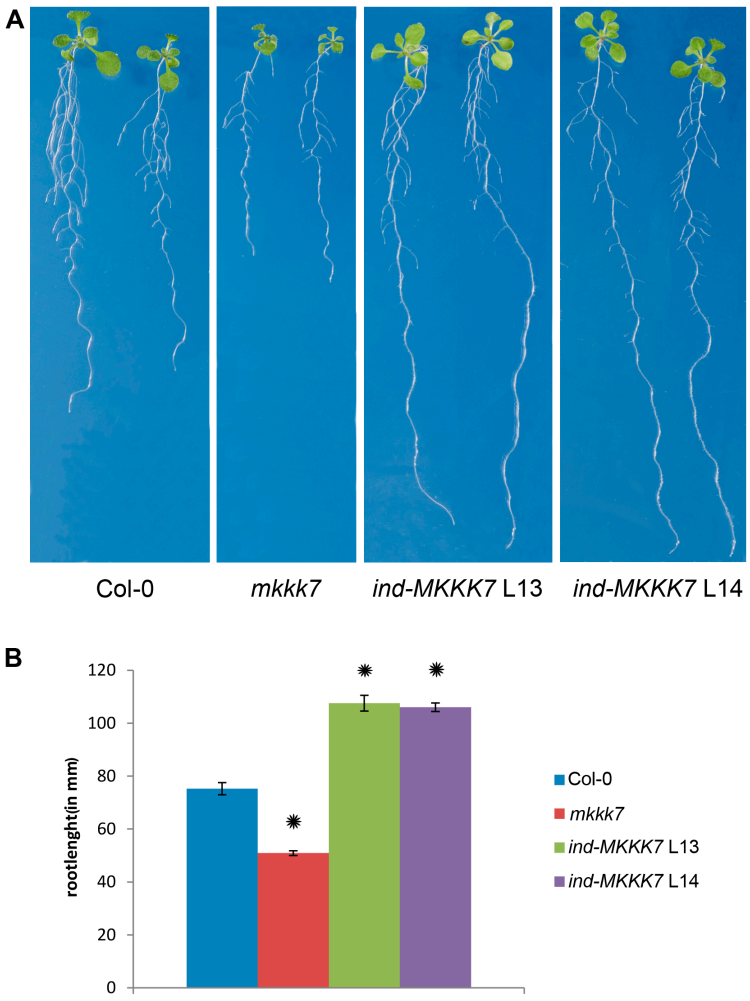

Figure 5. Inducible expression of *MKKK7* enhances root growth. **A)** Independent transgenic lines expressing *ind-MKKK7* grown on vertical half-strength MS agar plates show increased primary root growth. **B)** Measurement of root lengths of Col-0, *mkkk7, ind-MKKK7* independent line13 and line14 at 9 dpg. Data represents the mean values ± SEM (*n*=10; *, P<0.05; paired t-test). Experiment was repeated with similar results.

We also examined the meristem size in the transgenic lines at 48 to 72 hours post induction. A significant increase in meristem size of the *ind-MKKK7* lines was observed as compared to a Col-0 control (Fig. 6A).

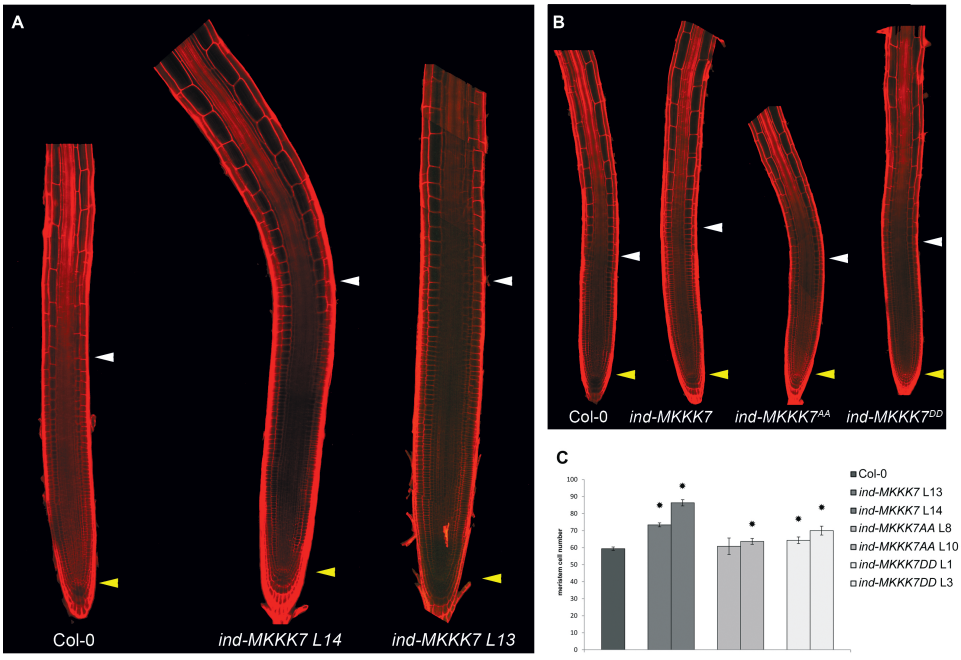

Figure 6. Inducible expression of *MKKK7* increases the root meristem. **A)** Confocal image of the meristem of seedlings expressing *MKKK7* after 48-72 h of estradiol induction. **B)** Confocal images of root meristems of seedlings induced 24 h with estradiol expressing either *MKKK7* (*ind-MKKK7*) or amino acid substituted versions of *MKKK7* (*ind-MKKK7^{AA}* and *ind-MKKK7^{DD}*) at 9 dpg, yellow arrowheads indicate the QC and white arrowheads indicate the upper boundary of the meristem where the cortex cells double in size. **C)** Quantification of number of meristematic cortex cells for different *ind-MKKK7* lines. The cortex cell number of 2 independently transformed lines for each *MKKK7* version was quantified. Data represents mean values ± SEM (*n*=4; *, P<0.05; paired t-test). Experiment was repeated with similar results.

We did not observe the isodiametric expansion of cells in the elongation zone. However we noticed a more gradual increase in cell length in an extended transition zone between meristem and elongation zone (Fig. 6A and 6B, left panel). Quantification of the number of cortex cells in the meristem of *ind-MKKK7* lines showed a significant increase in the number of cells in both transgenic lines after only 24 hours of induced *MKKK7* expression (Fig. 6B and 6C). We observed an enhancement of root length in the *ind-MKKK7* lines and a corresponding increase in root meristem size.

In Chapter 4, we demonstrated that two differentially phosphorylated serines (Ser) were required for full repression of disease resistance when *MKKK7* was overexpressed. To investigate the importance of phosphorylation of these differentially phosphorylated Ser for regulating root meristem size, we analyzed two independent inducible non-phosphorylatable *MKKK7^{AA}* lines (*ind-MKKK7^{AA}*) and two inducible phosphomimic *MKKK7^{DD}* transgenic lines (*ind-MKKK7^{DD}*). Confocal analysis of root meristems of these seedlings revealed little increase in cortex cell number of *ind-MKKK7^{AA}* line 8, showing a similar meristem size and cell number as Col-0, whereas *ind-MKKK7^{AA}* line 10 showed a very small but significant increase (Fig. 6C). Both *ind-MKKK7^{DD}* lines showed a small to moderate increase in meristem size and cortex cell number (Fig. 6C). All of the analyzed inducible overexpression lines had similar MKKK7:GFP protein levels (Fig. S2, bottom panel). These results show that controlled overexpression of *MKKK7* leads to increased meristem size due to enhanced of meristematic cell proliferation and this is largely dependent on the phosphorylation status of the previously identified Ser residues.

MKKK7 acts upstream of GLV/RGF peptides to control meristem size

Two of the phenotypes observed in the *OE-MKKK7* were recently described as overexpression phenotypes of genes encoding small secretory peptides called *Golven (GLV)* (Whitford et al., 2012). These GLV peptides were previously also described as RGF peptides (Matsuzaki *et al*., 2010). To test a possible connection between GLV/RGF and MKKK7 in relation to the regulation of the root meristematic zone, seedlings of Col-0 and *mkkk7* were treated with synthetic GLV/RGF peptides. We selected tyrosine sulfated versions of GLV5/RGF2 (GLV5*) and GLV11/RGF1 (GLV11*) which are expressed in the QC area and root cap and known to increase root meristem size (Matsuzaki *et al*., 2010) and used unmodified GLV1 peptide as a control treatment. Treatment with GLV1, which is expressed in flowers, hypocotyl, leaves and meristems, didn't result in enlargement of the root meristem. Treatment for 48-72 hours with GLV5* resulted in a moderately enhanced root meristem length only in *mkkk7* seedlings, while treatment with GLV11* induced a more pronounced increase in meristem size in both Col-0 and *mkkk7* (Fig. 7A). These observations were confirmed by quantification of the results after only 24 hours of treatment with peptides GLV1, GLV5*, GLV11* (Fig. 7B). When we tested higher concentrations of GLV5* and GLV11* both peptides could induce increases in meristem length in Col-0 and *mkkk7* (data not shown). Taken together these results show that GLV5*/RGF2* and GLV11*/RGF1* can rescue the *mkkk7* root meristem phenotype indicating that MKKK7 acts upstream of GLV/RGF peptides. This is consistent with the expression pattern of *MKKK7* in root tips, leaf margins, trichomes and developing ovules (Bedhomme *et al.*, 2009) and therefore MKKK7 may have a possible role in regulation of GLV/RGF peptides, which are involved in root meristem maintenance and meristem cell proliferation.

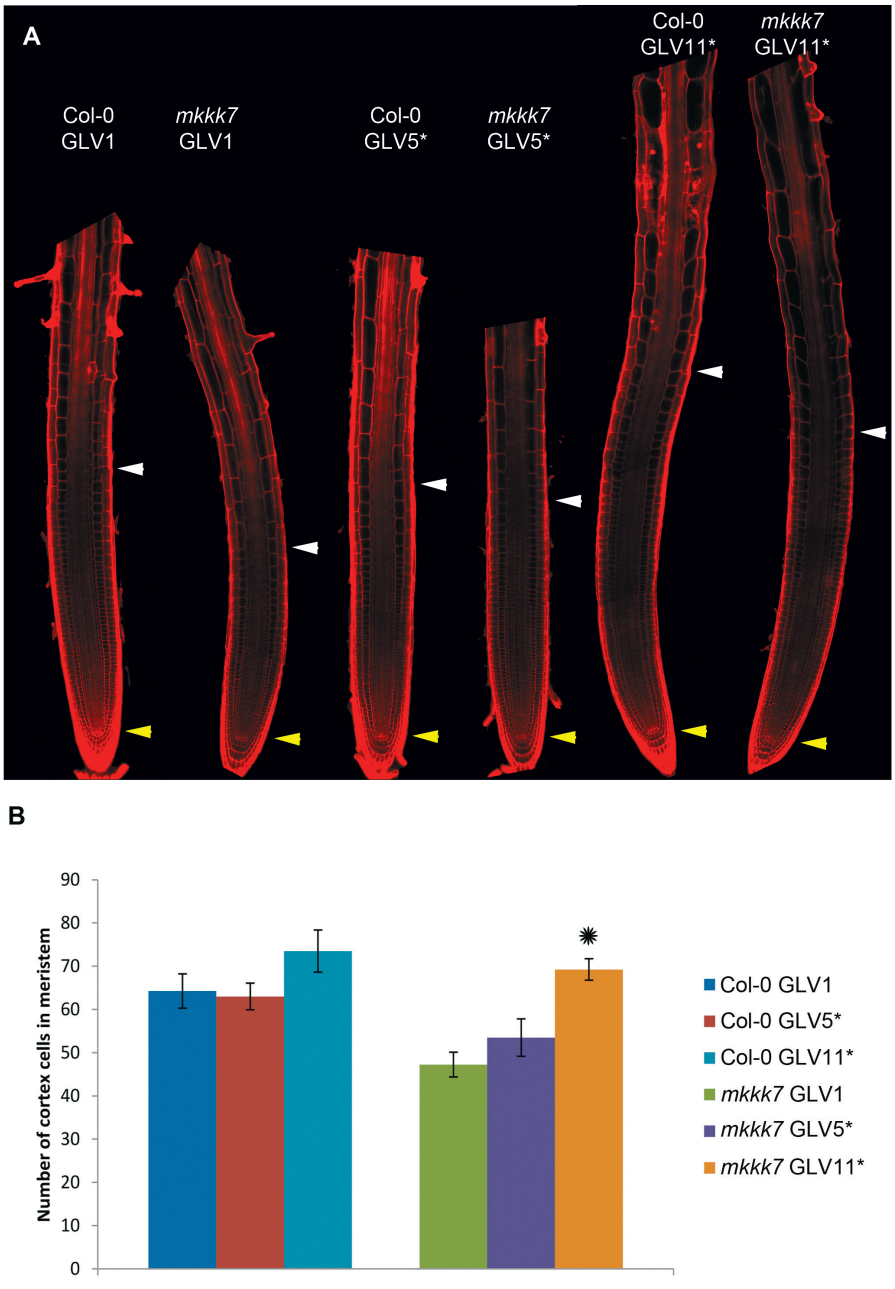

Figure 7. Tyrosine sulfated GLV/RGF peptides complement the *mkkk7* phenotype. A) Confocal image of the root meristem of Col-0 and *mkkk7* after treatment with 20 nM GLV1, GLV5*,GLV11* peptides for 48-72 h. **B)** Quantification of the number of cortex cells in the meristematic zone of Col-0 and *mkkk7* after treatment with 20 nM of GLV1,GLV5*,GLV11* peptides for 24 h. GLV1 is a non-sulfated glycopeptide and GLV5*and GLV11* are the tyrosine sulfated glycopeptides. Data represents mean values ± SEM (*n*=4; *, P<0.05; paired t-test).

DISCUSSION

In this study we provide evidence that MKKK7 has a role in the regulation of root proximal meristem maintenance. Complementation of the loss-of-function root phenotype of *mkkk7* with the *MKKK7:GFP* fusion (Fig. 3A and 3B) and results of the gain-of-function approaches (Fig. 4A and 4B) suggest that *MKKK7* is a positive regulator of the proximal meristem maintenance, which is an important aspect of root development. The only other MKKK with a clearly defined function in development is YODA, which acts as a key regulator of the stomatal pathway (Bergmann et al., 2004; Dong et al., 2010). The YODA MAPK cascade functions downstream of peptide cell-cell signaling to control meristemoid divisions and restricts the number of initial stomatal divisions (Dong et al., 2010).

Much less is known about signal transduction in response to peptide-mediated cell-cell signaling in the root apical meristem. Maintenance of root growth and root meristem size is a delicate balance between the rate of cell differentiation and cell division and involves a complex interplay of several plant hormones (Vanstraelen and Benková, 2012). The hormones cytokinin and auxin act antagonistically in feedback mechanism in the root transition zone to control the proximal root meristem size (Dello Ioio et al., 2008). We observed that in *mkkk7* the auxin induced expression of the early-auxin responsive gene *GH3* is reduced in seedlings (chapter 4, this thesis). Together with the reduction in meristem size this could indicate that the cytokinin-auxin interplay in the root of the *mkkk7* mutant is disturbed. However, exogenous application of auxin to *mkkk7* seedlings failed to rescue the reduced root growth (data not shown).

Constitutive or induced overexpression of *MKKK7* caused an enlarged root meristem and extended transition zone, indicating that *MKKK7* has a crucial role in regulating meristem size. The increase in root meristem size phenocopies the large meristems of cytokinin biosynthesis mutants, which were shown to be defective in regulating meristem cell differentiation (Dello Ioio et al., 2007). Both regulation of differentiation of meristem cells or regulation of meristematic cell proliferation could be modulated by *MKKK7*. Detailed expression analysis of cytokinin and auxin induced gene expression in *mkkk7* and *ind-MKKK7* lines will be required to provide more conclusive data whether *MKKK7* acts to modulate the cytokinin-auxin balance in the root apical meristem and by which possible mechanism.

The recent discovery of RGF peptides controlling root meristem size provides another link with the phenotype of *mkkk7* described in this chapter. The reduced meristem of *mkkk7* is similar to the phenotype of the *rgf1rgf2rgf3* mutant (Matsuzaki et al., 2010). Exogenous application of GLV5*/RGF2* and GLV11*/RGF1* could also rescue the *mkkk7* root proximal meristem phenotype (Fig. 7A) similar to the rescue by RGF1* described for *rgf1rgf2rgf3* (Matsuzaki et al., 2010). Furthermore, other phenotypes related to GLV/RGF peptide mediated responses were observed in constitutive overexpressors of *MKKK7* and included long root hairs, a significant increase in meristem cell proliferation and a wavy growing root (Fig. 4A). These phenotypes were also observed for overexpression of *GLV1/2/3* and exogenous application of μM concentrations of GLV1*/2*/3* (Whitford et al.,

2012). The treatment induces wavy root growth and was shown to be a consequence of *GLV*-mediated deregulation of auxin gradient formation causing a reduction in root gravitropism. Interestingly very low (nM) concentrations are sufficient to induce enlarged root proximal meristems without root waving (Matsuzaki et al., 2010; Whitford et al., 2012). In the *ind-MKKK7* we didn't observe strong defects such as irregular root waving including formation of loops, but the meristem size was enlarged. This could suggest that enlargement of the proximal meristem size can be uncoupled from deregulation of the auxin gradient formation and root agravitropism for both *MKKK7* overexpression and exogenous *GLV1*/2*/3** application. Thus, these results are in support of the observation that MKKK7 and GLV/RGF peptides act in the same pathway controlling several aspects of root meristem maintenance and root growth.

One of the first reports on the function of MKKK7 from Arabidopsis and its orthologue from *Brassica napus* also made a link with cell division (Jouannic et al., 2001). Partial complementation of *Schizosaccharomysis pombe* (*S.pombe)* mutant *cdc7* with *BnMKKK*□ was reported. It was suggested that MKKK7 and BnMKKK□ may function in cytokinesis similar to Cdc7 in the septum initiation pathway (SIN) in *S.pombe* (Jouannic et al*.,* 2001). The SIN pathway connects cytokinesis with mitosis, but no evidence supports that a similar pathway functions in plants (Bedhomme et al*.*, 2008). Furthermore, we also did not observe defects in cytokinesis in any of the mutant or overexpression lines, nor were they described for the *mkkk6 mkkk7* double mutant (Chaiwongsar et al*.*, 2006). Based on our results we postulate a different role for MKKK7 in the regulation of cell proliferation and not in cytokinesis. Similar conclusions were more recently also drawn by the authors of the original *cdc7* complementation paper (Bedhomme et al., 2008).

However, the very strong conservation of protein sequence and domains between MKKK7 and Cdc7 is striking, as shown in this chapter by phylogenetic analysis, amino acid sequence alignment and HMM domain analysis (Fig. 1C, Fig. S1). Plant homologues of Cdc7 all have an insertion into the ARM domains, effectively dividing this domain in two separate shorter ARM domains (Fig. 1C). Perhaps this insertion is associated with the recruitment of the plant Cdc7 homologues to a different aspect of cell division, which for MKKK7 could be control of cell proliferation in the root meristem. Other, closely related MKKKs belonging to the ANP family have been shown to be required for cytokinesis (Krysan et al*.,* 2002; Takahashi et al*.*, 2010; Beck et al*.,* 2011), however their phenotypes are not similar to the *mkkk7* phenotype.

Regulation of MKKK7 has hitherto not yet been reported. We provided evidence (chapter 4, this thesis and Fig. 6B and 6C) that the phosphorylation status of MKKK7 is required for its function. Overexpression of *MKKK7* resulted in a gain-of-function phenotype that affected root meristem size. This gain-of-function phenotype is largely dependent on the phosphorylation of two Ser residues and mutation of both residues reduced this phenotype. Induced expression of $MKKK7^{AA}$ has little effect on the meristem size whereas induced expression of $MKKK7^{DD}$ resulted in an intermediate gain-of-function phenotype. The fact that the phosphomimic mutant $MKKK7^{DD}$ has an intermediate phenotype is consistent with

previous observations that aspartate substitutions are not always as effective in mimicking a phosphorylated Ser or threonine residue. None the less, our results are in support with the requirement for phosphorylation of MKKK7 to positively regulate cell proliferations in the root apical meristem.

In conclusion our results suggest that MKKK7 has a function in root meristem maintenance, possibly through regulating proximal meristem cell divisions. We hypothesize that MKKK7 acts upstream of the specific RGF/GLV peptide family members, but cannot exclude that they act in a parallel pathway, required for the maintenance of the root stem cell niche and the proliferation of meristem cells that determine proximal root meristem size. Since RGFs regulate the expression of *PLT1* and *PLT2* transcription factors (Matsuzaki et al., 2010) future experiments will include approaches to gain insight in the connections between MKKK7 and the auxin regulated PLT pathway.

METHODS

Plant material

Arabidopsis thaliana accession Columbia (Col-0) was used. All mutant and transgenic lines used in this study were described previously in chapter 4, this thesis. The abbreviations of the used constructs are listed in supplemental table S1.

Bioinformatic analysis of MKKK protein sequences

Domain architectures for MKKK7 and *S. pombe* Cdc7 were obtained by submitting each full-length sequence to the HMMer "hmmscan" server (http://hmmer.janelia.org/search/hmmscan) (Finn et al., 2011), selecting Superfamily as the HMM database. The visual outputs of these two searches were combined to compare the domain architectures of the two sequences. Orthologs of *Arabidopsis* MKKK7 were retrieved from OrthoMCL-DB (http://www.orthomcl.org/) (Chen et al., 2006) as ortholog group OG5_131527. The orthologue sequences from several fungal species, including *S. pombe* and *Dictyostelium discoideum* were aligned with MAFFT version 6.935b (http://mafft.cbrc.jp/alignment/software/) (Katoh et al., 2005) and a gene tree was inferred from this alignment with FastTree version 2.1.4 (http://www.microbesonline.org/fasttree/) (Price et al., 2010) using the WAG substitution matrix, gamma model for rate variation and pseudocount option. Six sequences were selected, *Arabidopsis* MKKK7 and MKKK6, the single orthologue from *Oryza sativa*, the phylogenetically closest of three orthologs from *Physcomitrella patens*, *S. pombe* Cdc7 and *Dictyostelium discoideum* SEPA for further analysis. These sequences were aligned with T-Coffee version 8.99 (http://www.tcoffee.org/) (Notredame et al., 2000) using the PSI-COFFEE option to construct a highly accurate alignment and generate a colorized output alignment showing confidence scores for each aligned site.

Root length and root meristem size quantification

Harvested seeds were sterilized and stratified for 2 d at 4°C in the dark. Seeds were germinated on half-strength MS medium (Duchefa), containing 1.5% sucrose and 1% plant agar and the seedlings were grown in a vertical position in long day growth conditions, 16 h continuous light at 20-22°C. Root growth was registered every 24 h by marking the position of the root tip. The primary root length was measured using ImageJ (Fiji) software on calibrated photo's. Results presented are the calculated mean of 10 seedlings including the calculated standard error of mean (SEM). Root growth experiments were repeated at least two times with similar results. The root meristem size was determined as the number of cells in the cortex files extending from the quiescent center (QC) to the first elongated cell in the transition/elongation zone.

Nomarski microscopy

For quantification of the meristem size the roots of plants were cut, carefully positioned on a glass slide and cleared in chloral hydrate solution (50.0 mL water, 20 g chloral hydrate and 16 ml glycerol). The procedure was repeated for seedlings at several days up to 10 dpg.

Confocal imaging of roots

Root samples were counterstained with propidium iodide (PI), a nucleic acid binding dye that is excluded from the membranes of healthy cells. The PI staining is seen as a red fluorescent stain, surrounding each cell-outline in the root. The roots were observed under an inverted Zeiss confocal microscope laser, scanning with an Argon laser-neon with laser excitation 488 nm. Image analysis was done using Zeiss LSM Pascal (version 3.2 SP2) software.

Peptide treatment

Surface-sterilized *Arabidopsis* seeds of wild type Col-0 and *mkkk7* were germinated and grown on half-strength MS plates in a vertical position in long day growth conditions, 16 h continuous light at 20-22°C. After 7 dpg the seedlings were transferred for 24-72 h of peptide induction to 24 wells plates with liquid half-strength GM supplemented with synthetic peptides of GOLVEN GLV1 (At4g16515), GLV5 (At3g30350), GLV11(At3g30350). All peptides were dissolved in sterile sodium phosphate buffer (50 mM, pH 6.0), before dilution in 0.5*GM. The peptide sequences are described in more detail in Whitford et al. (2012). The used dose range of application was 20-100 nM unless otherwise specified. After 24-72 hours of incubation with different peptides, the meristem of the seedlings was imaged using confocal microscopy.

ACKNOWLEDGEMENTS

We thank Frits Kindt and Ronald Leito for photography and kind help for making the figures.

SUPPLEMENTAL DATA

Figure S1. Alignment of the protein sequences for orthologues of MKKK7. After a relation tree of MKKK7 orthologues was constructed, five orthologues of MKKK7 were selected as described in the methods section and the complete protein sequence was aligned. Below the alignment the consensus is shown. Abbreviations: *Arabidopsis thaliana* MKKK7 (Q9LJD8_ARATH) and MKKK6 (Q9SFB6_ARATH), *Oryza sativa* MKKK7 (Q01JL6_ORYSA), *Physcomitrella patens* MKKK7 (A9SNT8_PHYPA), *Schizosaccharomyces pombe* Cdc7 (CDC7_SCHIPO), *Dictyostelium discoideum* SEPA (SEPA_DICDI).

```
*
 BAD AVG GOOD
*
Q9LJD8_ARATH    :  95
Q9SFB6_ARATH    :  95
Q01JL6_ORYSA    :  95
A9SNT8_PHYPA    :  94
CDC7_SCHPO      :  96
SEPA_DICDI      :  96
cons            :  95

Q9LJD8_ARATH    MARQMTSSQFHKSKTLDNKYMLGDEIGKGAYGRVYK
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Q01JL6_ORYSA    MASRQHNAQFHKNKTLDNKYMLGDEIGKGAYGRVYK
A9SNT8_PHYPA    MSRHG-ASHFHKSKTLNDKYLLGDEIGKGAYGRVYK
CDC7_SCHPO      MHNIQ-----------ASSITLGDCLGKGAFGAVYR
SEPA_DICDI      MSKKE-PEEIKKNVTVG-NYNLGVVIGKGGFGTVYQ

cons            *                .  **  :***.:* **

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Q9SFB6_ARATH    GLDLENGDFVAIKQVSLENIGQEDLNTIMQEIDLLK
Q01JL6_ORYSA    GLDLENGDFVAIKQVSLENIPQEDLNIIMQEIDLLK
A9SNT8_PHYPA    GLDLDNGDFVAIKQVSLENIPSEDLASIMSEIDLLK
CDC7_SCHPO      GLNIKNGETVAVKKVKLSKMLKSDLSVIKMEIDLLK
SEPA_DICDI      GLDIEDGDFVAIKQINLTKIPKDQLQGIMNEIDLLK

cons            **:.:*: **:*::.* :: ..:*  *  ******

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Q01JL6_ORYSA    NLNHKNIVKYLGSLKTRSHLHIILEYVENGSLANII
A9SNT8_PHYPA    NLNHRNIVKYQGSFKTKTHLYIILEFVENGSLASII
CDC7_SCHPO      NLDHPNIVKYRGSYQTNDSLCIILEYCENGSLRSIC
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cons            **:* *****    :*.  * *:**: ***** .*

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A9SNT8_PHYPA    KPNKFGAFPESLVAVYIAQVLEGLVYLHEQGVIHRD
CDC7_SCHPO      K--NFGKIPENLVALYTFQVLQGLLYLHNQGVIHRD
SEPA_DICDI      K--KFGKFPETLVCVYIRQVLEGLVYLHEQGVVHRD

cons            *  :** :**.** :*  ***:**:***:***:***

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A9SNT8_PHYPA    IKGANILTTKEGLVKLADFGVATKLTEADINTHSVV
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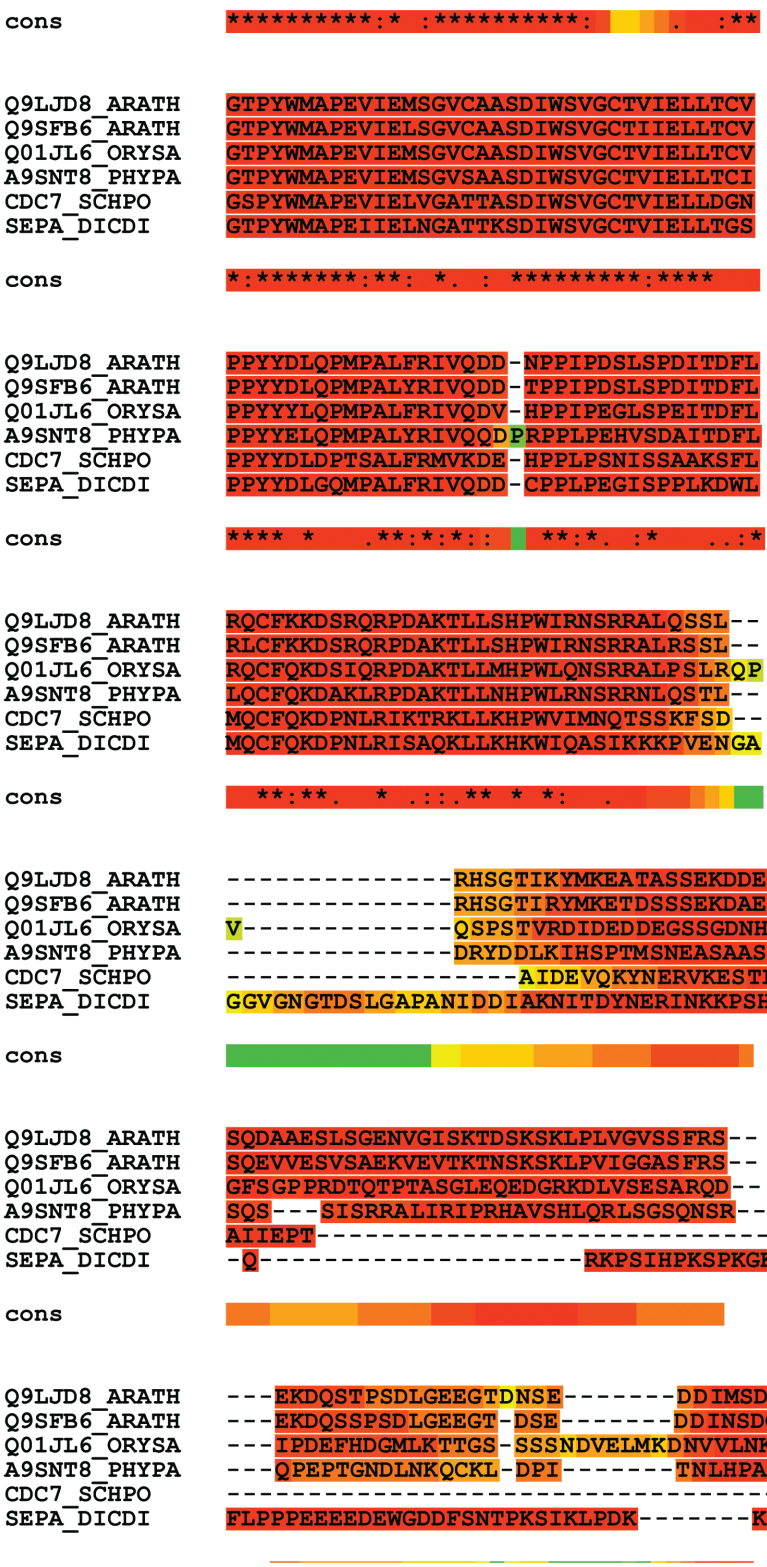
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cons **** * .**:*:*:: **:*. :* ..:*
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CDC7_SCHPO ------------------AIDEVQKYNERVKESTLT
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CDC7_SCHPO AIIEPT----------
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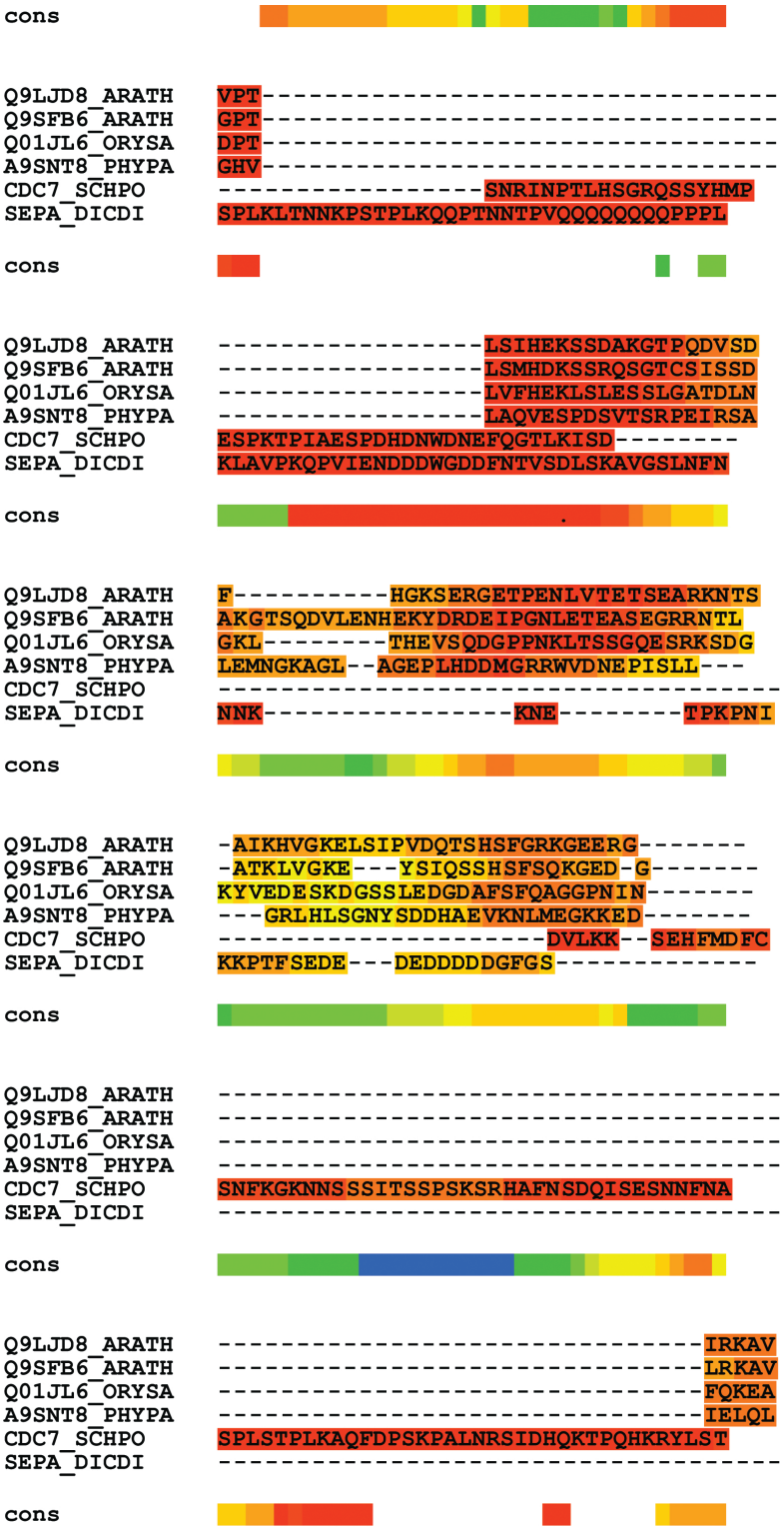
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SEPA_DICDI
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Q9SFB6_ARATH LRKAV
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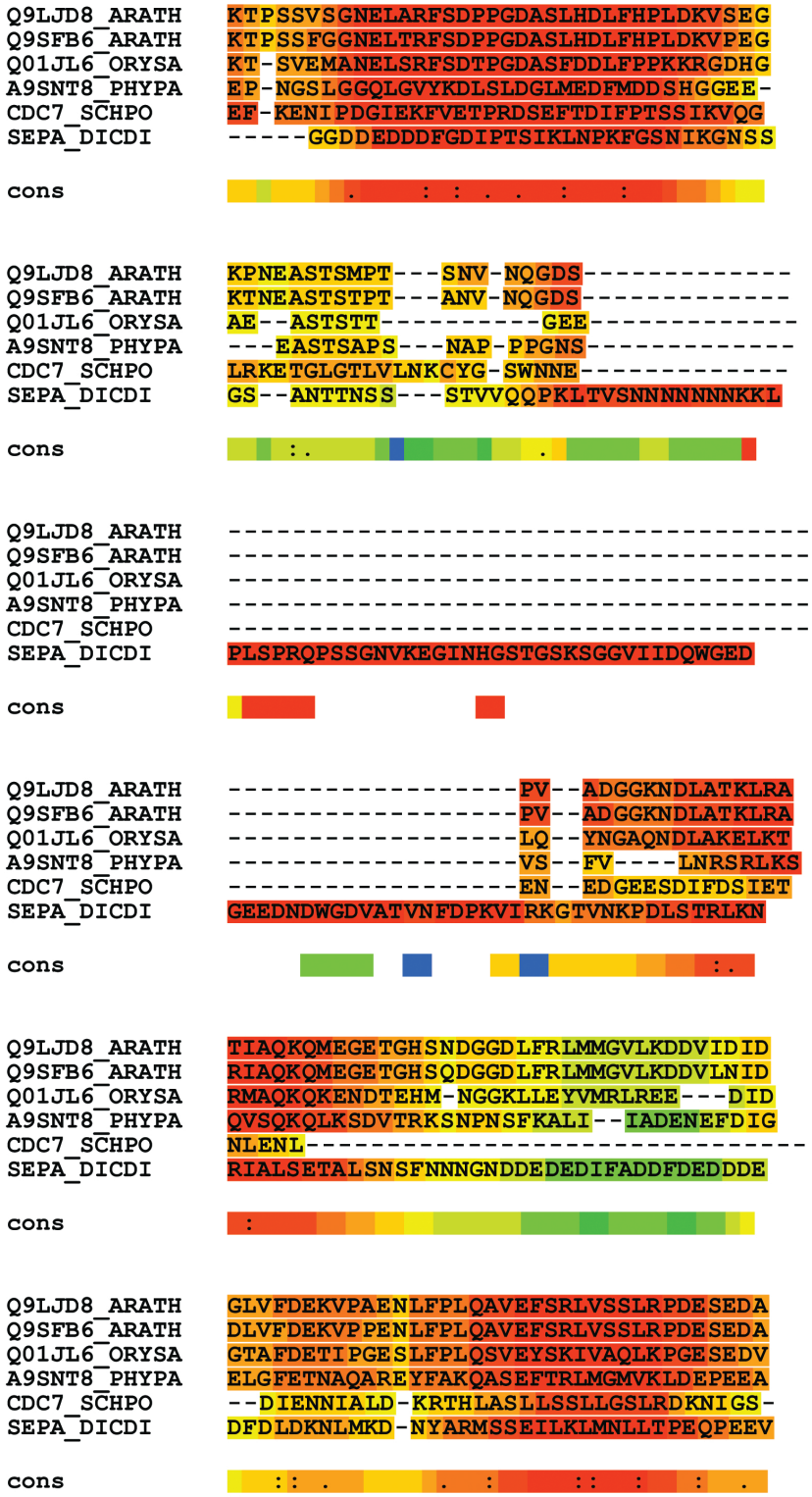
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CDC7_SCHPO --DIENNIALD-KRTHLASLLSSLLGSLRDKNIGS-
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cons

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CDC7_SCHPO      KDTTVSQIASILSEDLSLKREIIQAHGILPLLETLR
SEPA_DICDI      ISSACTQLITMFKENSEQKTLLIRRHGVIPIMEMLE

cons            .  .  ::  :: :    *   :  :*.:*::: *

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Q01JL6_ORYSA    LPK--NRIITSVLQLINQIVKDNTTFLENACLVGLI
A9SNT8_PHYPA    TSN--NRVLYEVLRVINHVIQDHVELQENACLIGLV
CDC7_SCHPO      EIK-TPDVQLLLLKLINTVAFDDHTTLQKVCFAGGL
SEPA_DICDI      VSNIQSHVLCSILKVVNQIIDNNMEIQENLCLVGGI

cons            :  :  :  :*:::* :  :.    :: *: * :

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CDC7_SCHPO      PLMLSFSNR--EHSFEFRYESAIFIQQMYRTSALTL
SEPA_DICDI      PAIMKFSGP--EYPASVRLETASFISKMCSTSTLTL

cons            * : .*:  :  :  . ..* : : *: ::  :*. **

Q9LJD8_ARATH    QMFIACRGIPVLVGFLEADYAKYREMVHLAIDGMWQ
Q9SFB6_ARATH    QMFISCRGIPVLVGFLEADYAKHREMVHLAIDGMWQ
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A9SNT8_PHYPA    QMFISCRGLPVLVGFLEPDYAKYREMVHMAIDGMWQ
CDC7_SCHPO      QMFLSSNGLNSLLLFIKEDYGTNRDFVFVGVEGIWK
SEPA_DICDI      QMFIACKGLPILVDFLLSPYAESKRLVWMAVDAIVN

cons            ***::..*:  *: *:   *.  : :. :.::.: :

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A9SNT8_PHYPA    VFDLQSSTSKNDFCRIFAKSGVLVRLVNTLHNLNEV
CDC7_SCHPO      LLRQQDYIPKNDICTMVVNDSLEPLTKAMLKAL---
SEPA_DICDI      VFELQSPTPKNDFCRLFSKCGLLKTLPIVLRDS---

cons            .:  :   .:**:* :  : .:      *

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CDC7_SCHPO      ------------------------------------
SEPA_DICDI      ------------------------------------

cons
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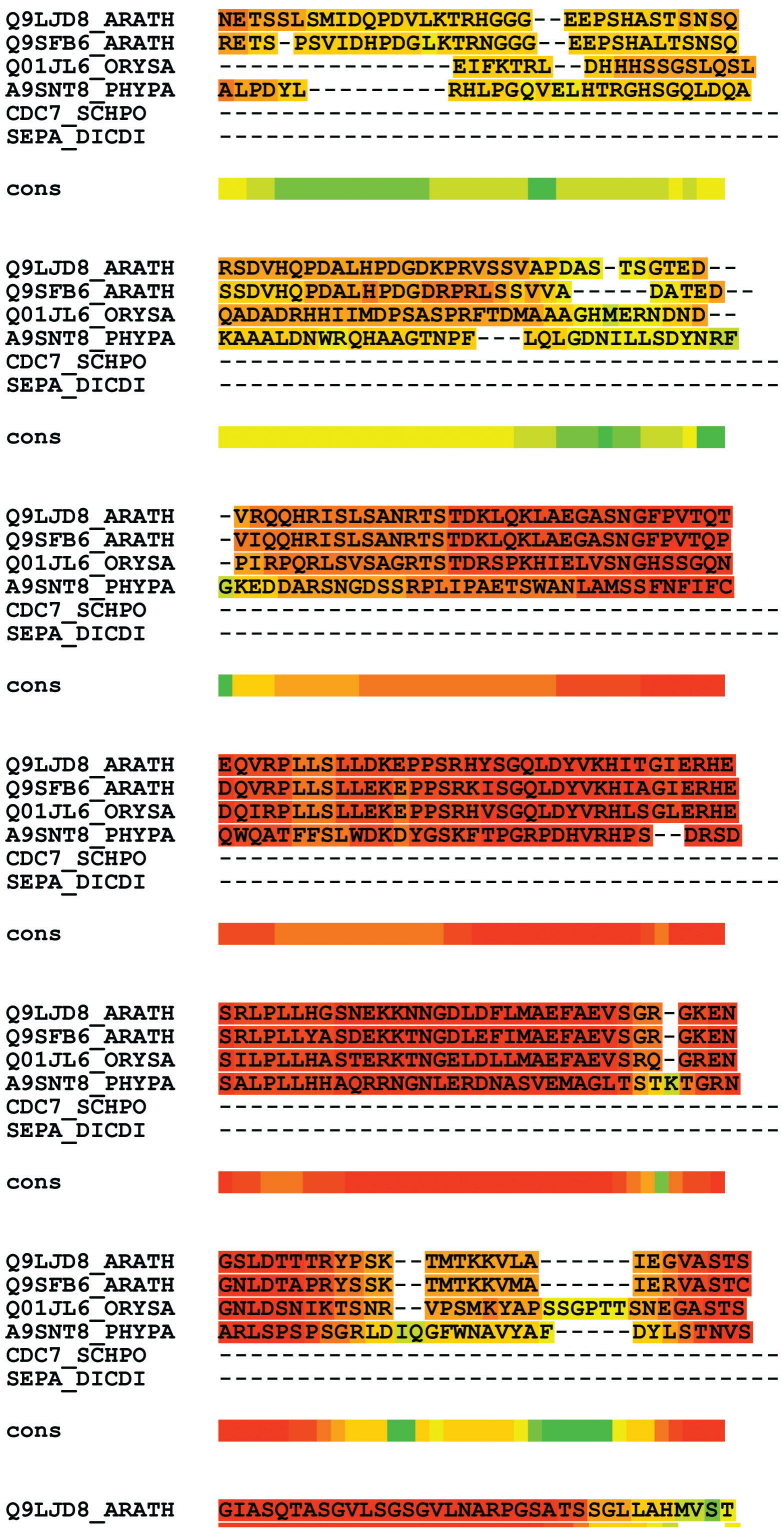
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CDC7_SCHPO ----------
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cons
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CDC7_SCHPO ----------
SEPA_DICDI ----------
cons
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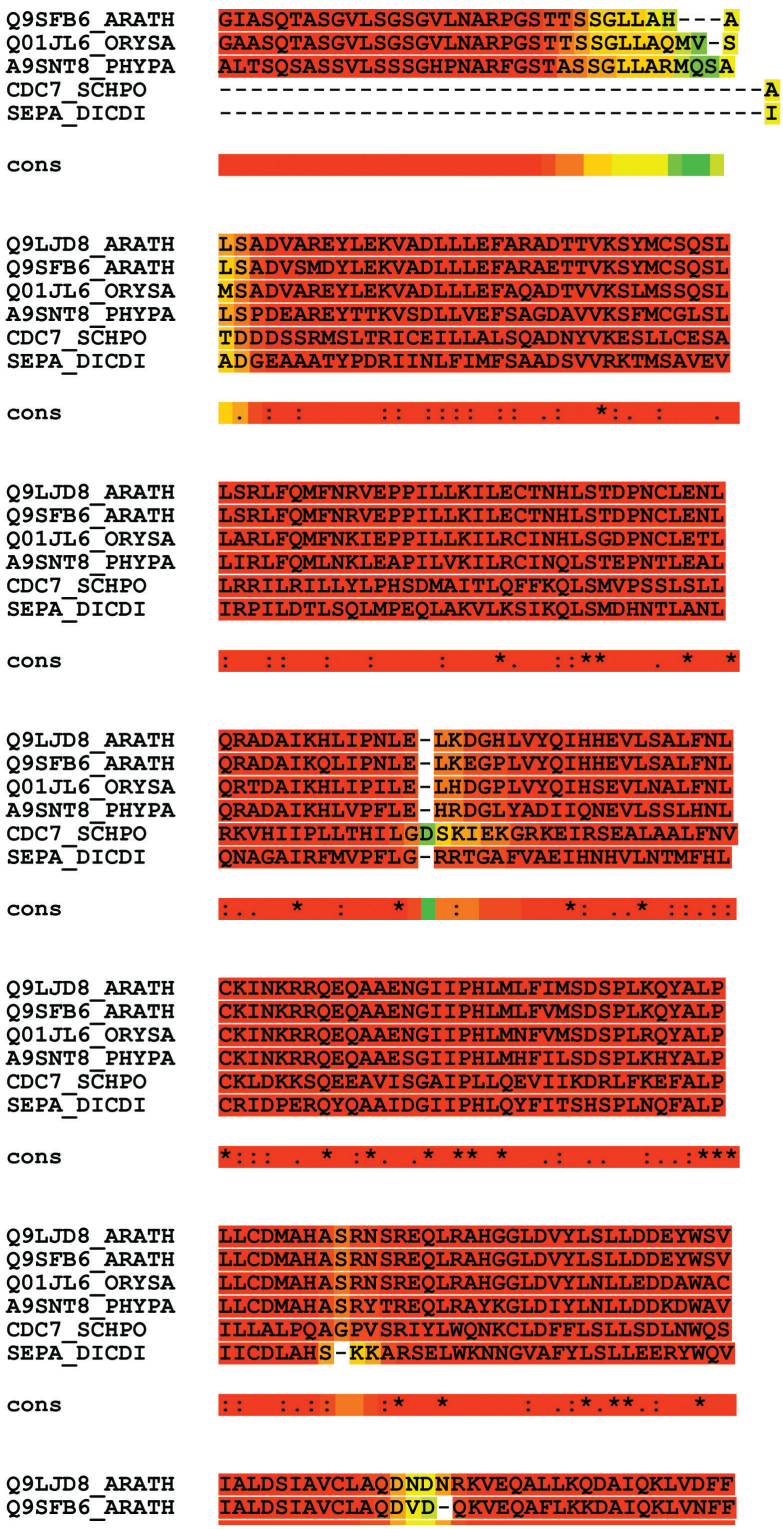
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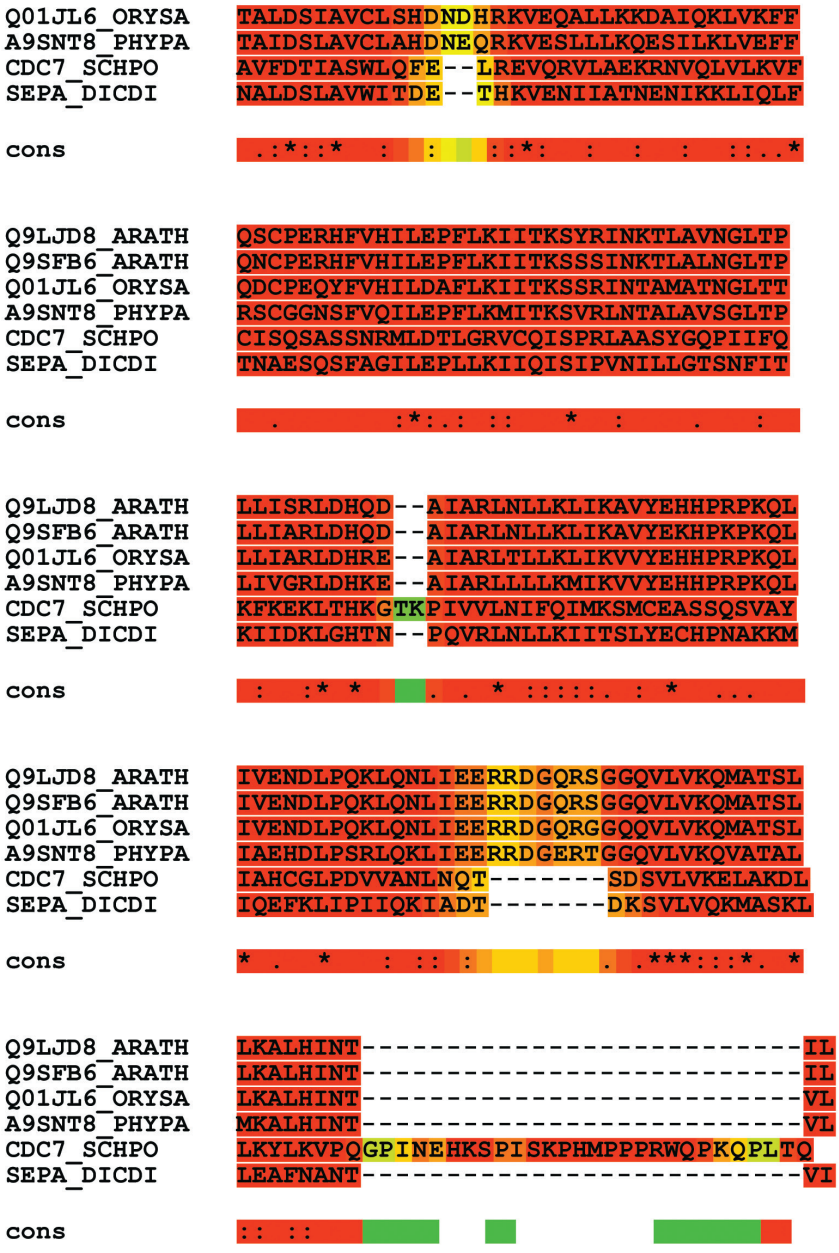
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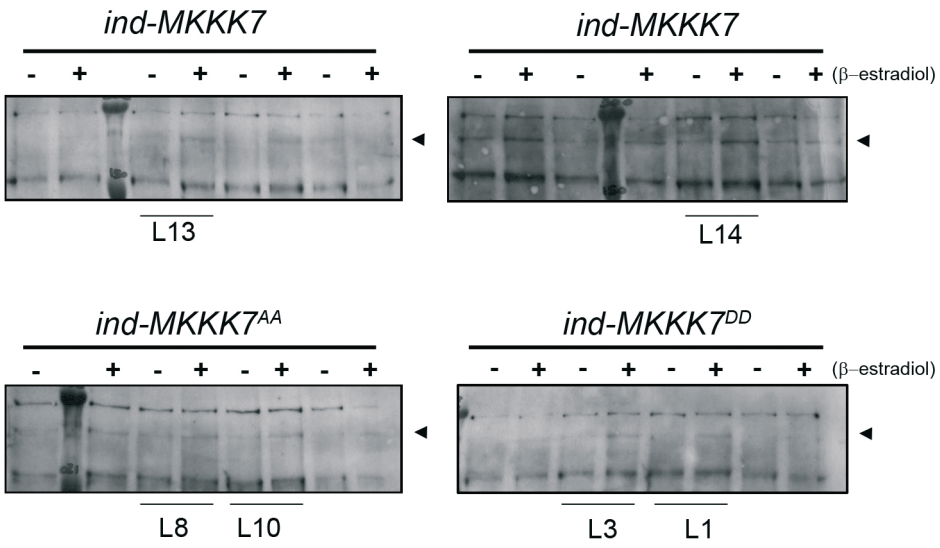

Figure S2. **Western blot analysis for inducible *MKKK7* seedlings**. The levels of MKKK7:GFP expression were visualized with an α-GFP antibody. Position of MKKK7:GFP in the blots is indicated with arrowheads. The indicated independent T3 lines indicated below each panel were selected for follow-up analysis.

Supplemental table S1: constructs of *MKKK7* for complementation in plants.

Purpose	Construct	Abbreviation
Complementation in plants	*35S::MKKK7:GFP*	*OE-MKKK7*
	pMKKK7::MKKK7:GFP	Not abbreviated
	pG1090::XVE UAS::MKKK7:GFP	*ind-MKKK7*
	pG1090::XVE UAS::MKKK7$^{SS->AA}$:GFP	*ind-MKKK7^{AA}*
	pG1090::XVE UAS:MKKK7 $^{SS->DD}$:GFP	*ind-MKKK7^{DD}*

CHAPTER 6

6

Summarizing Discussion

PROTEIN PHOSPHORYLATION IN PLANT DEFENSE SIGNALING

Plants are notable for their ability to adapt to their ever-changing environment through modulation of endogenous developmental programs. This requires integration of environmental cues at the cellular level to affect transcriptional reprogramming of cells within the responding tissue (Osmont et al., 2007). Locally, cells can respond to cell-cell signaling, which includes peptide signaling mediated by receptor-like kinases that transduce an endogenous (self) signal intracellularly to modify transcriptional regulation of developmental responses (Katsir et al., 2011). Similarly, cells can respond to exogenous cues (non-self) at the cellular level through plasma membrane localized pathogen recognition receptors (PRRs) that initiate a signaling cascade leading to transcriptional defense responses (Segonzac and Zipfel, 2011). These intracellular signaling cascades frequently rely on the reversible covalent modification of pre-existing transducer molecules and can amplify the signal (Pawson and Scott, 2005; Schulze, 2010; Mithoe and Menke, 2011). The prototypic modification involved in intracellular signaling in eukaryotic cells is protein phosphorylation (Pawson and Scott, 2005).

While about a third of all proteins is thought to exist in a phosphorylated form, phosphoproteins with regulatory functions and involved in signal transduction are generally low abundant and show sub-stoichiometric levels of phosphorylation (Choudhary and Mann, 2010). This makes the analysis of signaling events particularly challenging. The rapid advances in mass spectrometric technology have opened the possibility to analyze the specific sites of phosphorylation of proteins with regulatory functions at a proteome-wide scale in model organisms with a sequenced genome, including a growing number of model plant species (Benschop et al., 2007; Nuhse et al., 2007; Grimsrud et al., 2010). Interpreting the biological relevance of phosphorylation sites is aided by recent advances in quantitative proteomics approaches (Schulze and Usadel, 2010). However experimental verification through functional analysis of candidate phosphoproteins is still required.

In this thesis different approaches to analyze the role of the phosphoproteome are described during pathogen-associated molecular pattern (PAMP) triggered signaling in Arabidopsis. In particular, approaches to investigate the modulation of components of the conserved mitogen-activated protein kinase (MAPK) signaling cascade downstream of the flagellin receptor FLS2 have been undertaken. We combined novel proteomics approaches and an in-depth functional analysis to investigate the function of phosphorylation of different MAPK cascade members after PAMP recognition and we linked phosphorylation of one of the targets, MAPK kinase kinase 7 (MKKK7) to antagonistic regulation of basal immunity and root growth and development.

In **Chapter 3,** a directed shotgun phosphoproteomics approach is described to selectively measure and quantify tyrosine (Tyr) phosphorylation in plant cells (Mithoe et al., 2012). In general, the analysis of Tyr phosphorylation can be a challenging quest, due to the low levels of phosphorylated Tyr (pTyr) which are between 0.5%-3% of the total phosphoproteome (de la Fuente van Bentem and Hirt, 2009; Mithoe and Menke, 2011). By

comparison, the levels of phosphorylated threonine (pThr) contribute about 15%-20% and phosphorylated serine (pSer) about 70%-75% of the phosphoproteome. This may explain why initial studies of the plant phosphoproteome that used suboptimal phosphopeptide enrichment methods, detected mostly peptides phosphorylated on Ser and Thr residues (Nuhse et al., 2004; Benschop et al., 2007; de la Fuente van Bentem et al., 2008). This technical problem may have been confounded by the fact that protein phosphorylation is very rapid and transient in nature and signaling proteins such as kinases are generally low abundant. We used a single step immunoprecipitation (IP) based enrichment of pTyr containing peptides (Boersema et al., 2010) and identified 139 Tyr phosphorylated sites (**chapter 3)**. The largest proportion of these pTyr peptides were derived from proteins annotated as protein kinases, which was consistent with previous observations (Nakagami et al., 2010). Within this set, two families of Tyr phosphorylated kinases, the glycogen synthase kinase 3 (GSK3 or SHAGGY-related kinase) and MAPKs were overrepresented. Both these classes require phosphorylation of their activation loop for kinase activity.

MAPKs comprise the largest set of identified Tyr phosphorylated proteins downstream of the FLS2 receptor

By combining the $^{14}N/^{15}N$ metabolic labeling method (Engelsberger et al., 2006; Benschop et al., 2007) with a pTyr IP, we were able to study the changes in the pTyr proteome in a reproducible system and measured differentially phosphorylated Tyr peptides after perception of the 22-amino acid peptide flg22, derived from the bacterial PAMP flagellin. FLS2-mediated perception of flg22 is the paradigm for PAMP-triggered immunity (PTI) signaling in plants and elicits a wide range of pathogen responses in plants (Nicaise et al., 2009). Signal perception occurs at the plasma membrane and is mediated through a receptor–like kinase complex of FLS2-BAK1, which initiates a signaling cascade, triggers an oxidative burst within minutes and activates several downstream kinase targets including MPK3, MPK4 and MPK6. Activation of these MAPKs has been connected to early PAMP signaling and defense related responses (Asai et al., 2002; Menke et al., 2004; Pitzschke et al., 2009; Rodriguez et al., 2010). In our screen we identified this set of the known PAMP-activated MAPKs (MPK3, MPK4/11 and MPK6) as highly differentially phosphorylated on the Tyr residue of the conserved T-x-Y motif.

Additionally, another set of MAPKs (MPK8, MPK9, MPK13 and MPK17) was identified with enhanced Tyr phosphorylation after flg22 induction, which had not been connected to flg22-mediated PTI signaling before. Out of this set, MPK13 belongs to group B MAPKs, which also includes MPK4 and MPK11, while MPK8, MPK9 and MPK17 belong to the enigmatic group D MAPKs. Of this latter group D MAPKs, only MPK8 has been described and is activated through mechanical wounding and requires binding of calmodulins (CaMs) and MKK3 phosphorylation. MPK8 mediates Ca^{2+} and reactive oxygen species (ROS) signaling in early wound responses to ensure an appropriate concentration of ROS (Takahashi et al., 2011). With the recent identification of MPK11 as a fourth PAMP-triggered MAPK (Bethke

et al., 2012; Eschen-Lippold et al., 2012) and our data on differential Tyr phosphorylation of four additional MAPKs in response to flg22, the phosphorylation-dependent regulation of MAPK signaling has become more complex than hitherto appreciated.

Interestingly, in response to flg22 perception MPK6 was dually phosphorylated on both the Tyr and Thr residue in the activation loop. The dual phosphorylation on both residues correlates with the strong activation of MPK6 within minutes after stimulation with flg22, peaking at 5-10 minutes (Mithoe et al., 2012). The MPK3 activation loop could not be shown to be dually phosphorylated and only peptides phosphorylated on the Tyr residue were identified (Mithoe et al., 2012). Extensive *in vivo* studies of MPK6 and MPK3 have demonstrated that both these MAPKs are highly activated after flg22 induction (Asai et al., 2002; Benschop et al., 2007; Suarez-Rodriguez et al., 2007; Bethke et al., 2012; Mithoe et al., 2012) and therefore peptides phosphorylated on both Thr and Tyr should occur for MPK3. The most likely explanation for the absence of the dually phosphorylated peptide is a technical explanation. The MPK3 dual phosphorylated peptide is a longer peptide and might not make a good precursor ion during mass spectrometric analysis, precluding its selection for fragmentation required for its identification by shotgun proteomics. We were able to detect dually phosphorylated peptides for MPK4, which is a negative regulator of defense-related responses (Petersen et al., 2000) and was also shown to be activated by flg22 (Bethke et al., 2012; Mithoe et al., 2012), but these observations were done in one biological replicate and need more observations for definite confirmation.

In a recent follow-up targeted proteomics approach using selective reaction monitoring (SRM), we reproducibly detected dually phosphorylated peptides for MPK3, MPK4/11 and MPK6 in flg22-induced samples prepared in an identical manner (Frank Menke, unpublished data). Therefore, it is likely that the difficulty in detecting the dually phosphorylated peptides for the other 6 MAPKs by shotgun proteomics is due to a combination of low protein levels and characteristics of the dually phosphorylated peptides that make it suboptimal precursor ions. We can infer that all of the 7 MAPKs detected as differentially phosphorylated on the Tyr residue in response to flg22, are derived from at least 7 flg22-activated MAPKs. This highlights a much more complex MAPK phosphorylation cascade, which will require extensive functional analysis, including the generation of double or triple mutant combinations to circumvent redundancy in MAPK signaling. This could be done by using artificial microRNAs (amiRNAs) that are an effective tool for specific gene silencing in plants, especially when several related, but not identical target genes need to be down-regulated. With this RNA silencing method, the problem of lethality when generating double mutants can be avoided (Schwab et al., 2006).

Interestingly none of the kinases of the related GSK3 family were identified as differentially phosphorylated in response to flg22. It was recently shown that autophosphorylation/dephosphorylation of the GSK3-like kinase BRASSINOSTEROID INSENSITIVE2 (BIN2) on Tyr200 is a critical switch in the downstream regulation of brassinosteriod signaling, which is mediated by the BRI1 receptor-like kinase (Kim and Wang, 2010; Jaillais et al., 2011). FLS2 and BRI1-mediated signaling both require the (BRI1)-

ASSOCIATED KINASE1 (BAK1) co-receptor for signaling and this has raised the question whether BRI signaling and FLS2 signaling show cross regulation (Belkhadir et al., 2011; Albrecht et al., 2012). Recently this issue has been addressed by these two separate studies, which both indicate that while FLS2-mediated signaling can be suppressed by high levels of BRI1-mediated signaling, FLS2 signaling does not regulate brassinosteroid responses. Our observation, that GSK3 is not differentially phosphorylated by flg22 perception supports these conclusions. A separate interaction involving GSKs and MAPK signaling is described in stomatal development. The GSK-3 BIN2, involved in the negative regulation of BRI-mediated signaling, also acts on the YODA (YDA) MAPK cascade regulating initial stomatal linage divisions (Kim et al., 2012). Low levels of BRI signaling result in BIN2-mediated phosphorylation of YDA, while high levels of BRI signaling inhibit BIN2 activity (via regulation of the autophosphorylated pTyr200) to release YDA from the negative regulation and to allow MAPK signaling required for appropriate stomatal patterning. It will be interesting to see whether similar regulation by BRI signaling also regulates MKKK activity in the FLS2 cascade. With this single step pTyr enrichment approach we gained more insight into an extraordinary complexity of MAPK signal transduction pathways in Arabidopsis and specificity of the FLS2 signaling cascade. We also independently confirmed that Tyr phosphorylation in Arabidopsis occurs at levels similar to metazoan model systems, which had remained controversial after the initial reports by the Shirazu group (Sugiyama et al., 2008; Nakagami et al., 2010).

In metazoan model systems Tyr phosphorylation has gained particular attention and has been suggested to play a specific role in signal transduction. Selective tyrosine kinases and phosphatases, that add and remove phosphoryl groups to tyrosine residues, together with specific pTyr binding domains have been postulated to constitute a specific signaling module, the so-called reader, writer and eraser system (Lim and Pawson, 2010). This module, which includes receptor tyrosine kinases (RTKs) that are involved in many important cellular functions, such as proliferation, differentiation, cell survival and metabolism (Lemmon and Schlessinger, 2010; Lim and Pawson, 2010), is absent in the plant kingdom. In metazoan systems Tyr phosphorylation plays diverse roles at the molecular level, in enzyme activation/deactivation, protein localization and degradation and includes proteins with a signaling function as well as metabolic enzymes (Lim and Pawson, 2010). Plant phosphoproteomic data suggests that the pTyr containing proteins are overrepresented in signaling proteins, in particular protein kinases (Sugiyama et al., 2008; Grimsrud et al., 2010; Nakagami et al., 2010; Mithoe et al., 2012). Further characterization of the pTyr proteome in more plants species and under a wide range of conditions and tissue types will be required to prove whether this generalization for Tyr phosphorylation will hold true for the plant phosphoproteome.

TAKING A LOOK INTO THE BLACK BOX

Functional analysis of MKKK7

We next examined the role of phosphorylation at the level of MAPKKKs in innate immunity, which has remained a black box in extracellular receptor kinase-mediated signal transduction. The initial identification of differential phosphorylation at the MKKK level was performed using plant cell cultures and a large set of early-signaling proteins was identified as modulated by PAMP perception, including a set of MAPKKKs and RAF-like kinases (Benschop et al., 2007).

In **chapter 4**, the functional analysis of one of the modulated MKKKs is described and by using a loss-of-function and a gain-of-function approach we demonstrate that Arabidopsis MKKK7 is required for the attenuation of defense responses and negatively regulates basal immunity. We also provide evidence that this attenuation of defense responses depends on the phosphoregulation of MKKK7. Detailed analysis of the downstream signaling events suggest that, MKKK7 acts through modulation of MPK3, MPK4 and MPK6, as has been shown by a reduction to the basal activity level of these MPKs in response to flg22 treatment in inducible MKKK7 overexpression mutants (**chapter 4**). Remarkably, at the same time phosphoprotein MKKK7 seems to be a genuine positive regulator of root meristem cell proliferation, which is required for root meristem maintenance and root growth, as described in **chapter 5**.

We provide evidence for the importance of phosphorylation of MKKK7 on the Ser residues in the antagonistic regulation of basal immunity and root growth and root meristem length. The functional analysis of MKKK7 illuminates the complexity of the role of MAPKKKs in MAPK signal transduction pathways and also reinforces the notion that phosphorylation of individual MAPK cascades members, including MKKKs, is important for the regulation of their activity.

Connecting MKKK7 to downstream MAPK members

In Arabidopsis PTI signal transduction, complete pathways involving MPK3 and MPK6 remain elusive. We provided evidence that MKKK7 is a negative regulator of basal immunity in **chapter 4** and attenuates MPK3, MPK4 and MPK6 activity in response to flg22 induction. We were unable to identify the downstream MEK component of this cascade, but there is another example of a complete cascade negatively regulated by the RAF-like kinase, constitutive triple response 1 (CTR1). This kinase was shown to inhibit the MKK9-MPK3/MPK6 module, which regulates the stability of the EIN3 protein in response to ethylene. The negative regulation of the MKK9-MPK3/MPK6 cascade was shown by genetics and has not been proven to be direct (Yoo and Sheen, 2008; Bethke et al., 2009; Yoo et al., 2009; Bisson and Groth, 2011).

In addition to the biochemical evidence that MKKK7 negatively regulates MPK3, MPK4 and MPK6 (**Chapter 4**, Mithoe and Menke, unpublished data) we also noted during

the course of generating transgenic lines evidence for a genetic interaction. Inducible-overexpression lines of *MKKK7* in wildtype background did not show an embryonic phenotype, such as aberrant number of cotyledons or defective embryonic roots. When transformed into a background in which both MPK6 and MPK3 are silenced by RNAi (*MPK6i3i*), a significant percentage revealed a range of phenotypes in the T1 generation. We observed that 25% of the T1 generation had severe developmental phenotypes showing either fused cotyledons, single cotyledons, tri-cotyledons or a lack of an embryonic root (Mithoe and Menke, unpublished data).

We previously observed similar numbers of embryo defects in selected double mutant combinations in an allelic series for *mpk3* and *mpk6* null alleles. It is not possible to create a double null mutant of *mpk6* and *mpk3,* however in *mpk6*$^{-/-}$*mpk3*$^{+/-}$ seedlings, 25% of the seedlings had a severe phenotype that was either rootless or had aberrant cotyledons. This haploinsufficient phenotype was also noted in relation to the female gametophyte (Wang et al., 2008). None of the other allelic combinations showed such a penetrant phenotype and the next strongest allelic combination was the *mpk6* mutant by itself, with 10% aberrant seedlings as noted before (Mithoe and Menke, unpublished data; (Wang et al., 2007)). The *MPK6i3i* line did not show this aberrant phenotype at a percentage higher than wildtype (less than 1%; Mithoe and Menke, unpublished data).

A possible explanation is that introduction of an overexpression construct for *MKKK7* in *MPK6i3i* results in enhanced penetrance of the embryonic phenotype to the level of the haploinsufficient *mpk6*$^{-/-}$*mpk3*$^{+/-}$ combination. This suggests that (leaky) *MKKK7* expression in *MPK6i3i* can further reduce the activity of MPK6 to a level equivalent to the *mpk6* null allele, recreating the aforementioned haploinsufficient situation. This data implies a genetic interaction between MKKK7 and MPK3 and MPK6, in support of our biochemical data.

Does enhanced defense signaling in *mkkk7* indicate a primed state?

Plant immune signaling is connected via complex interactions with almost the entire spectrum of phytohormones to ensure a proper balance between immune signaling, plant growth and development (Bari and Jones, 2009). Since immune responses, growth and development both require significant amounts of energy, it is important to attenuate defense responses under normal growth conditions when plants are not under pathogen attack. We presented evidence in **chapter 4**, that MKKK7 is one of the molecular components of a system that controls the attenuation of immune signaling and suppresses basal immunity. The mutant *mkkk7* showed enhanced defense gene expression of *WKRY29* and *FRK1* in protoplasts after flg22 treatment. Additionally, an increase in phosphorylation status of MPK6, and to a lesser extent MPK3 and MPK4 was observed in seedlings of *mkkk7* after treatment with flg22. Mutant *mkkk7* plants also show enhanced resistance to virulent pathogen *Pst* DC3000. Increased pathogen or PAMP-triggered defense gene expression and enhanced resistance are the hallmarks of a primed status, which can be induced in plants chemically or through

the perception of non-host pathogens or non-pathogenic microbes (Prime-A-Plant Group., 2006; Conrath, 2011).

The exact molecular mechanism of priming has long been a mystery, but recently several theories have been put forward. One of these theories suggests that priming of cells could involve accumulation of inactive cellular proteins in anticipation of post-translational modification and activation as was shown for MPK3 and MPK6 (Beckers et al., 2009). The ENHANCED DISEASE RESISTANCE 1 mutant (*edr1*), showed enhanced resistance to bacterial and fungal pathogens (Frye et al., 2001; Tang et al., 2005). In the constitutively primed *edr1* mutant there was no need for a priming-inducing treatment to strongly activate MPK3 and MPK6 protein activity. Interestingly, in the priming deficient *npr1* (Non-expressor of PR1) mutant, accumulation and enhanced activation of MPK3 and MPK6 was attenuated (Beckers et al., 2009). Moreover, priming and systemic immunity were absent in *mpk3*, but not completely compromised in *mpk6*. Together this data leads to the assumption that MPK3 is a major molecular component in primed defense gene activation and systemic immunity in Arabidopsis, while MPK6 likely serves only a minor role (Beckers et al., 2009; Conrath, 2011).

Our data is consistent with a primed status of *mkkk7* and we provided biochemical evidence that suggests enhanced activation of MPK6 (**chapter 4**), which we recently confirmed by a quantitative SRM approach on flg22-induced *mkkk7* seedlings (Menke et al., unpublished data). To determine how MKKK7 regulates MPK3 and MPK6 in priming defense related responses, analyzing the effect of double mutant combinations of *mkkk7* with *mpk3*, *mpk6* as well as *npr1* could shed more light on this issue. Also testing the effectiveness of chemical-induced and rhizobacteria-mediated priming leading to induced systemic resistance should be tested in *mkkk7* and inducible overexpressing MKKK7 lines. In addition, it would be interesting to verify the expression levels of marker genes associated with systemic immunity such as *PAL1* and *PR1* in *mkkk7*.

Phosphorylation of MKKK7 is required to attenuate immune response and enhances root meristem growth

In **chapter 4** we investigated the importance of phosphorylation of MKKK7 in suppression of PAMP-induced defense gene expression and basal immunity. Overexpression of *MKKK7* suppressed flg22-induced inhibition of root growth, flg22-induced defense gene expression and enhanced susceptibility to *Pst* DC3000. When we analyzed inducible transgenic lines with both differentially phosphorylated Ser residues mutated into non-phosphorylatable alanine (*ind-MKKK7*^{AA}), the suppression of flg22-induced defense gene expression, as well as enhanced susceptibility were not observed. Conversely, in inducible phosphomimic *MKKK7*^{DD} (*ind-MKKK7*^{DD}) transgenic lines, flg22-induced defense gene expression was completely suppressed. Also, the amount of disease symptoms after *Pst* DC3000 infiltration of the *ind-MKKK7*^{DD} lines was significantly more than observed in Col-0 and both *ind-MKKK7*^{AA} lines, although it did not reach the level observed in the *ind-MKKK7* lines. These observations are

in line with the requirement of phosphorylation of MKKK7 at one or both Ser residues to effectively attenuate defense response and basal immunity.

We also observed that modulation of *MKKK7* expression affected root growth and root meristem size (**chapter 5)**. Mutant *mkkk7* displayed a shorter root phenotype and controlled overexpression of *MKKK7* lead to an increased meristem size due to enhanced meristematic cell proliferation**.** Therefore, we also examined the importance of phosphorylation of the differentially phosphorylated Ser residues for regulating root meristem size. Two independent transgenic lines of *ind-MKKK7^{AA}* did not show enlarged root meristems upon induced expression, whilst at least one of the two independent transgenic of *ind-MKKK7^{DD}* showed an enhanced proximal root meristem. Taken together these results demonstrate, that positive effect of overexpressing *MKKK7* on the size of the root meristem is largely dependent on the phosphorylation status of *MKKK7*. It is not known what the exact molecular mechanism of phosphoregulation of these Ser residues is, however substitution analysis revealed that this unknown function is required for the antagonistic regulation of immune response and root growth. It may well be that single amino acid substitution versions of MKKK7 could shed light on some of the more complicated phenotypes, observed during analysis of the loss-of function and gain-of-function *MKKK7* lines.

MKKK7 AS A POINT OF CROSSTALK BETWEEN GROWTH AND DEFENSE?

The antagonistic function of MKKK7 is connected to tyrosine sulfated peptide signaling

In **chapter 5** we provided data supporting that MKKK7 functions as a positive regulator of the root meristem. The short-root phenotype and reduced proximal root meristem displayed by the *mkkk7* mutant is restored by overexpressing *MKKK7.* Inducible overexpression of *MKKK7* causes an enlarged meristem, which we could link to the phosphorylation status of MKKK7. The phenotypes described in **chapter 5** are also associated with a set of secreted peptides called root growth factors (RGFs) or GOLVEN (GLV), which are involved in root meristem maintenance (Matsuzaki et al., 2010; Whitford et al., 2012). Plant peptides are known to be involved in stem cell homeostasis (Stahl and Simon, 2009, 2012) and recent research has described a family of secreted tyrosine sulfated peptides with a role in cell proliferation and meristem maintenance (Matsubayashi and Sakagami, 1996; Amano et al., 2007; Matsuzaki et al., 2010). This includes a growth-promoting peptide, phytosulfokine (PSK), which was recently described to be involved in the attenuation of PTI signaling in *Arabidopsis* (Igarashi et al., 2012; Mosher et al., 2012). Homozygous loss-of-function mutant PSK receptor (*pskr1*) showed enhanced defense gene expression and seedling growth inhibition triggered by PAMPs, similar to *mkkk7*.

Tyrosyl protein sulfotransferase (TPST) is an enzyme involved in catalyzing the trans-sulfation of the precursors of PSK to generate mature PSK. The *tpst-1* mutant exhibited enhanced PAMP-triggered seedling growth inhibition. Interestingly *tpst-1* and *mkkk7* both

have a shorter root meristem and in **chapter 5** we observed that the reduced proximal root meristem of *mkkk7* is restored by adding tyrosine sulfated versions of GLV5/RGF2 (GLV5*) and GLV11/RGF1 (GLV11*). The *tpst-1* mutant can be rescued by adding PSK in combination with RGF1, whilst PSK or RGF1 alone partially rescue the root phenotype of *tpst-1* (Matsuzaki et al., 2010). The enhanced resistance to infection with *Pst* DC3000 of *tpst-1* is also partially rescued by the addition of PSK (Mosher et al., 2012) and a similar experiment should be performed to test whether the enhanced resistance of *mkkk7* is also reduced by RGF application.

However, recent observations described by Igarashi et al. (2012) and Mosher et al. (2012) on the antagonistic regulation by phytosulfokines on root meristem maintenance and resistance to biotrophic bacterial pathogens suggest a strong correlation with the antagonistic regulation by phospho-MKKK7. The fact that RGF1 and RGF2 are able to complement the root phenotype of *mkkk7* is in line with these observations and our results suggest that MKKK7 acts upstream or in parallel to TPST, PSKR1 and RGF-mediated signaling. Further tests with exogenous application of PSK and RGFs to *mkkk7* in root meristem assays as well as resistance tests to *Pst* DC3000 can elucidate this extend of correlation. Additionally, genetic analysis such as overexpression of MKKK7 in the *tpst-1* background should confirm whether MKKK7 acts (partially) upstream of the tyrosine sulfate peptide signaling, which is important for the balance between cell proliferation and attenuation of PTI (Fig. 1).

In relation to the antagonistic regulation of meristem proliferation and basal immunity to biotrophic bacterial pathogens it is interesting to consider the role auxin plays here as well. PAMP-triggered immunity has been shown to repress auxin signaling, via miRNA mediated downregulation of auxin receptors (Navarro et al., 2006). Furthermore several biotropic plant pathogens are known to produce auxin which suppresses host defense responses (Robert-Seilaniantz et al., 2007; Robert-Seilaniantz et al., 2011a). Auxin mediated suppression of basal immunity acts through counteracting salicyclic acid (SA) biosynthesis and SA signaling (Robert-Seilaniantz et al., 2011b), which is required during PTI to restrict growth of biotrophic pathogens. By contrast, necrotrophic pathogens which are resisted mainly though jasmonic acid (JA)-dependent defense responses, are more effective in infecting plants compromised in auxin signaling. In plants with compromised tyrosine sulfated peptide signaling, such as *tpst-1, pskr1* and *psy1r* mutants, immunity to necrotrophic pathogen *Alternaria brassicicola* is reduced (Mosher et al., 2012). This set of mutants was also recently shown to be more resistant to biotrophic pathogen *Pst* DC3000 and it is likely that an imbalance in auxin signaling causes this antagonistic regulation of biotrophic versus necrotrophic immunity (Mosher et al., 2012). GLV/RGF peptides, have been connected to both the biosynthesis as well as the polar transport of auxin (Matsuzaki et al., 2010; Whitford et al., 2012). We noted that in *mkkk7* application of exogenous RGF/GLV peptides could rescue the meristem size phenotype.

Furthermore, we also observed that the auxin-induced expression of the early-auxin responsive gene GH3 is reduced in *mkkk7* seedlings, which indicates compromised auxin signaling (**chapter 4**). Since *mkkk7* mutants are also more resistant to *Pst* DC3000,

we speculate that MKKK7 acts upstream of RGF signaling and that mutations in *MKKK7* compromise RGF signaling and auxin response. This results in reduced meristem proliferation and enhanced immunity to biotrophic pathogen *Pst* DC3000. Future experiments may include analyzing the gene expression levels of marker genes for the JA and SA signaling pathways, such as *PDF1.2, OPR3* and *PR1* in *mkkk7* and *ind-MKKK7* lines and might elucidate the changes in the balance between SA signaling and JA dependent signaling. The susceptibility for *Alternaria brassicicola* in *mkkk7* and resistance in *ind-MKKK7* plants, as well as measuring the SA and JA content should also be tested. This will contribute to the insight, whether MKKK7 acts though tyrosine sulfated peptide signaling and whether activated responses are modulated through antagonistic JA-SA signaling pathways.

In conclusion we identified a mechanism in which PAMP-triggered cues guide plant growth, acting though modulation of the activity of MKKK7. The main function of MKKK7 is to maintain the antagonistic balance between regulation of cell proliferation in the primary root meristem and attenuation of MPK3 and MPK6 dependent defense-related signaling. This intertwined regulation provides the plant with a powerful capacity to finely regulate its immune responses against the pathogen encountered and to utilize its resources in a cost-efficient manner. Maintenance of the balance between plant growth and immunity contributes to fitness of the plant in varying environmental conditions.

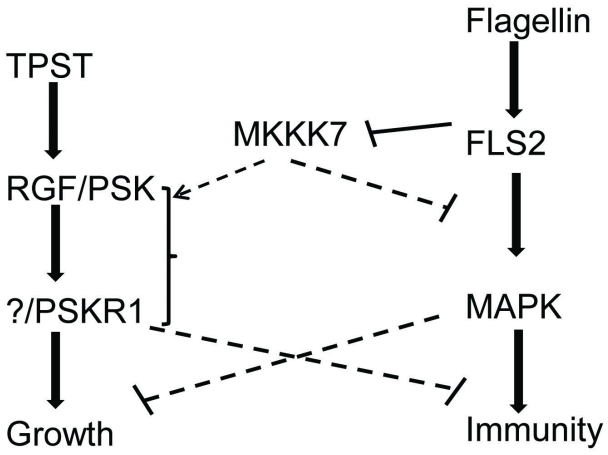

Figure 1. Hypothetical model for the regulation of phosphoprotein MKKK7 in maintenance of the balance between root growth and attenuation of PTI. PTI signaling and tyrosine sulfated peptide signaling mutually antagonize downstream responses (adapted from Igarashi et al., 2012). In the absence of flagellin perception, MKKK7 may be involved in the positive regulation of growth through tyrosine sulfated peptides including RGF/PSK (dotted arrow). These peptide growth factors function as ligands for an unknown receptor and PSKR1. At the same time MKKK7 attenuates PTI through antagonistic signaling downstream of the PAMP receptor FLS2 and is therefore involved in shifting resource allocation between growth and immunity. Future investigation of molecular mechanisms involved in RGF/PSK/PSKR1 signaling will contribute to our understanding of how different biological processes are coordinated to optimize the fitness of the plant. The model is adapted from Igarashi et al. (2012).

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SAMENVATTING

Planten bezitten het karakteristieke vermogen om zich aan te passen aan hun steeds veranderende omgeving. Mede door hun gebrek aan mobiliteit moeten ze in staat zijn om zich in extreme condities te verweren tegen abiotische stress, zoals hitte, droogte, kou en overstromingen. Ze moeten ook biotische stress, waaronder aanvallen van microbiële ziekteverwekkers, kunnen weerstaan en overleven. Het uitbreken van plantenziekten heeft directe gevolgen voor de wereldvoedselproductie en onderzoek naar afweermechanismen in planten kan dus een belangrijke bijdrage leveren aan de voedselveiligheid. De meest effectieve afweer wordt gevormd door passieve natuurlijke barièrres zoals een waslaag aan de buitenkant, huidmondjes op de bladeren die zich kunnen sluiten, een dikke celwand en het afscheiden van anti-microbiële stoffen. Daarnaast bezitten planten ook een basaal immuunsysteem dat in staat is om "lichaamsvreemde" stoffen te detecteren net zoals bij mensen en dieren. Dit zorgt ervoor dat een potentiële microbiële ziekteverwekker wordt herkend en een snelle afweerreactie wordt geactiveerd om dit pathogeen geen kans te geven zich te verspreiden. Het is hierbij van belang dat de juiste interne signaaltransductieroutes worden gemoduleerd, zodat de plant zich op een efficiënte manier kan verweren zonder daarbij onnodig energie te verspillen.

Modificaties van eiwitten, de zogenaamde post-translationale modificaties (PTMs), zijn belangrijk voor snelle en specifieke veranderingen van eiwitten die betrokken zijn bij deze signaleringsroutes. Eiwitfosforylatie is een essentieel aspect voor de regulatie en signalering en fosforylering vindt voornamelijk plaats op de aminozuren Serine (Ser), Threonine (Thr) en Tyrosine (Tyr). Kinasen, enzymen die andere eiwitten fosforyleren, spelen hierbij een belangrijke rol en worden zelf ook vaak gereguleerd door middel van fosforylatie. Zo ook de mitogen-geactiveerde proteine kinasen (MAPKs), die een centrale rol spelen bij plantenafweer en ontwikkeling. De MAPKs zijn onderdeel van een module van drie eiwitten die functioneel met elkaar verbonden zijn en na herkenning van een potentiële ziekteverwekker elkaar sequencieel fosforyleren, hetgeen zorgt voor activatie van deze eiwitten en ook voor de versterking van het signaal. De gefosforyleerde MAPKs kunnen vervolgens een skala van afweergerelateerde eiwitten fosforyleren die nodig zijn voor een adequate een afweerrespons.

In hoofdstuk 2 wordt een literatuuroverzicht gegeven van de recente ontwikkelingen om modificaties van eiwitten in de cel ("phosphoproteomics") te meten in planten. De toename in gevoeligheid van metingen maakt het technisch ook mogelijk om de Tyr fosforylering in complex cel- materiaal te meten. Van Tyr fosforylering is bekend dat het een belangrijke rol speelt tijdens de ontwikkeling en afweer in onder andere dierlijke modelsystemen. Recent is er ook meer aandacht voor de rol van Tyr fosforylering in planten gekomen, door de ontwikkeling van specifieke proteomics methoden. Naast de vaak toegepaste shotgun proteomics aanpak, worden deze nieuwe methoden belicht die toegepast kunnen worden om meer inzicht te krijgen in de veranderingen van belangrijke signaaltransductie eiwitten in planten.

Het in dit proefschrift beschreven onderzoek centreert zich rond het ontrafelen van de PTMs, die in gang worden gezet na herkenning van het kort stukje eiwit afkomstig van flagelline (flg22), een bouwsteen in het flagellum van pathogene bacteriën. Voor dit onderzoek is het plantje *Arabidopsis thaliana* (zandraket) als modelsysteem gebruikt. Tijdens de snel geïnduceerde afweerrespons veroorzaakt door toevoeging van flg22, speelt Tyr fosforylatie een belangrijke rol voor de activering van MAP kinasen.

Het onderzoek in hoofdstuk 3 beschrijft een gedetailleerde analyse van Tyr fosforylatie van laag abundante signaleringseiwitten, die betrokken zijn bij een immuunrespons na toevoeging van flg22. Uit voorgaande studies is gebleken dat fosforylatie van Tyr technisch moeilijk te meten is en dit wordt onder andere veroorzaakt door de relatief lage frequentie van Tyr fosforylatie en de lage abundantie van eiwitten betrokken bij signaleringsprocessen. Door het combineren van twee nieuwe methoden, de zogenaamde metabole labeling en specifieke verrijking van gefosforyleerde Tyr residuen was het mogelijke Tyr fosforylatie reproduceerbaar te meten. Voor deze studie zijn plantencellen gebruikt, die op ^{15}N stabiel isotopen medium zijn gegroeid en vervolgens behandeld werden met flg22, gevolgd door een verrijking van de fractie van Tyr gefosforyleerde eiwitten met een speciek antilichaam. Met behulp van massaspectrometrie zijn er 135 Tyr gefosforyleerde eiwitten gedetecteerd, waarvan in 11 eiwitten een verhoogde fosforylatie op de Tyr residu werd gemeten na vergelijking met de onbehandelde ^{14}N controle cellen. Het gebruik van ^{15}N en ^{14}N gelabelde plantencellen maakt een directe vergelijking door middel van massaspectrometrie mogelijk.

Naast de identificatie van bekende 'spelers' betrokken bij pathogeen-geïnduceerde responsen zoals MPK3, MPK4, MPK11 en MPK6 werden er nog vier MAP kinasen MPK8, MPK9, MPK13 en MPK17 gevonden, die nog niet eerder in verband waren gebracht met flg22-geïnduceerde afweer. MAPKs kunnen op basis van overeenkomsten in sequentie worden geclassificeerd in groep A, B, C (met een TEY motief) en groep D (met een TDY motief). MPK3 en MPK6 die geclassificeerd zijn in groep A, reguleren de immuunrespons in positieve zin. In tegenstelling tot groep B, waartoe MPK4, MPK11 en MPK13 behoren, die een negatieve rol spelen bij de afweerrespons na blootstelling aan microbiële componenten zoals flg22. MPK8, MPK9 en MPK17 behoren tot de groep D MAPKs, waarvan er heel weinig over de functie bekend is. In deze studie werd er geen verhoging van Tyr fosforylatie gevonden van eiwitten betrokken bij andere signaleringsroutes, zoals glycogen synthase kinase 3 (GSK3), die betrokken is bij brassinosteroid signalering. De beschreven observaties geven aan, dat signalering via de flagelline gevoelige receptor (FLS2), specifieke signaleringsroutes aanschakelt die tot een afweerreactie leiden. De bevindingen suggereren ook dat MAPK signaaltransductie en in het bijzonder de flg22-geïnduceerde afweer een hoge mate van complexiteit bevat, die nog vele jaren onderzoek vraagt om voldoende inzicht te krijgen hoe dit belangrijke netwerk van eiwitten gereguleerd wordt.

Het onderzoek in hoofdstuk 4 beschrijft een gedetailleerde analyse van de functie van een mitogen-geactiveerde kinase kinase kinase 7 (MKKK7). Na herkenning van een ziekteverwekker door de receptor FLS2 wordt een cascade van MAPK eiwitten geactiveerd, beginnend met een MAP kinase kinase kinase (MAPKKK). Deze eiwitten

activeren door fosforylatie de zogenaamde MAP kinase kinasen (MAPKK) die op hun beurt de MAPKs fosforyleren en activeren. De MAPKs fosforyleren dan vervolgens een skala aan afweergerelateerde eiwitten waardoor de signalering wordt doorgezet en tot een afweerreactie leidt. In Arabidopsis zijn zo'n 80 MAPKKK eiwitten geïndentificeerd, maar er is slechts van enkele MAPKKK eiwitten een functie beschreven. MKKK7 is geïdentificeerd in een eerder uitgevoerde phosphoproteomics studie van onze groep, waarbij er minder fosforylatie van twee Ser residuen werd gemeten na toevoeging van flg22. Om meer inzicht in de functie te krijgen werden *MKKK7* gemuteerde planten (*mkkk7*) behandeld met flg22 en dit leidde tot een verhoogde afweerrespons in het bladmateriaal van mutant planten. Op eiwitniveau werd dit resultaat bevestigd door een verhoging in de fosforylatie van MPK3, MPK4 en MPK6 na toevoeging van flg22. Daarintegen, in transgene zaailingen met induceerbare overexpressie van *MKKK7* (*ind-MKKK7*) is na toevoeging van flg22 een zeer sterke reductie te zien in MPK3 en MPK6 fosforylering en MPK4 wordt zelfs niet meer geactiveerd. Hieruit kon worden afgeleid dat MKKK7 een negatieve regulator is van flg22-geïnduceerde MAPK activatie. Om inzicht te krijgen hoe MKKK7 de immuunrespons in planten reguleert, werden *mkkk7* en *ind-MKKK7* planten ook behandeld met de bacteriële ziekteverwekker *Pseudomonas syringae* pv. tomato (*Pst* DC3000). Planten met verminderde expressieniveaus van MKKK7 vertonen minder ziektesymptomen na blootstelling aan *Pst* DC3000. De *ind-MKKK7* planten waren veel zieker na behandeling met *Pst* DC3000 in vergelijking met controle plantjes. Deze resultaten suggereren dat MKKK7 niet alleen zorgt voor de onderdrukking van MAPK activatie, maar ook van afweerresponsen en daarmee als een negatieve regulator van de basale immuniteit kan worden gekarakteriseerd.

Om te onderzoeken of fosforylatie van de eerder geïndentificeerde Ser residuen een rol spelen voor de functie van MKKK7 werden beide Ser vervangen door het aminozuur alanine (MKKK7^{AA}), waardoor een niet-fosforyleerbare versie van MKKK7 werd gecreëerd. Door de Ser residuen met het aminozuur aspartaat (MKKK7^{DD}) te substitueren, werd een variant van MKKK7 gegenereerd die constant gefosforyleerd lijkt. In protoplasten (cellen zonder celwand) met *MKKK7^{AA}* expressie was de afweerrespons na flg22 behandeling vergelijkbaar met de controle. Ook in transgene *ind-MKKK7^{AA}* planten was er geen significant waarneembaar verschil te zien met de controle plantjes na blootstelling aan het pathogeen *Pst* DC3000. Maar in protoplasten met *MKKK7^{DD}* overexpressie is een significante reductie in afweergenexpressie te zien en dit duidt op een vermindering in afweerrespons na behandeling met flg22. Ook transgene *ind-MKKK7^{DD}* planten die blootgesteld werden aan *Pst* DC3000 vertoonden meer ziektesymptomen in vergelijking met de controle planten. Deze observaties suggereren dat fosforylering van één of allebei geïndentificeerde Ser residuen van MKKK7 essentieel is voor het onderdrukken van de afweermechanismen en de basale immuniteit in Arabidopsis. Verder onderzoek zal moeten uitwijzen op welke manier de fosforylatie van MKKK7 zijn functie exact beïnvloedt.

Naast de rol die MKKK7 speelt bij de afweerrespons tegen potentiele microbiële ziekteverwekkers, werd ook een wortelgroeifenotype waargenomen in planten zonder functioneel MKKK7 eiwit (*mkkk7)*. In hoofdstuk 5 wordt aangetoond dat modulatie van *MKKK7*

expressieniveaus effect heeft op de primaire wortelgroei en de daarmee samenhangende grootte van het wortelmeristeem (de groeipunt van de wortel). Door herstel van het oorspronkelijke expressieniveau van *MKKK7* in de *mkkk7* mutant achtergrond (middels complementatie met een transgen bestaande uit de promoter en het coderende deel van *MKKK7*), observeerden we een herstel in groei van de wortel en het wortelmeristeem. In transgene planten met *MKKK7* overexpressie werd een sterke toename van de lengte van het primaire wortelmeristeem waargenomen. Na gedetailleerde bestudering bleek deze toename van het wortelmeristeem het resultaat te zijn van een toename in het aantal cellen in het primaire meristeem en niet de lengte van de cellen in het meristeem. Fosforylatie bleek belangrijk voor deze functie van MKKK7. Het aantal cellen in wortel met verhoogde expressie van $MKKK7^{AA}$ was vergelijkbaar met de controle plantjes, maar bij overexpressie van $MKKK7^{DD}$ was er significante toename van het aantal cellen in het wortelmeristeem en de bijbehorende toename in lengte van de primaire wortel. Deze observaties duiden erop dat ook voor een normale groei van de wortel fosforylering van één of beide Ser residuen essentieel is. De MKKK7 wortelmeristeemfenotypen leken op fenotypen die eerder waren beschreven voor een groep gemodificeerde signaleringspeptiden betrokken bij wortelgroei, de zogenaamde 'root growth factors' (RGFs) of 'Golven' (GLV). Toevoegen van sommige van deze RGFs bleek de groei van de korte *mkkk7* wortel te kunnen herstellen naar de wortellengte en meristeemgrootte van controle wortels. Deze bevindingen suggereren dat MKKK7 een functie heeft bij het in stand houden van het wortelmeristeem en dat dit mogelijk gebeurt door regulatie van de celdellingen in het proximale wortelmeristeem zoals eerder beschreven werd voor de RGF peptiden.

In hoofdstuk 6 worden tot slot de resulaten van het uitgevoerde onderzoek bediscussiëerd en samengebracht. De beschreven observaties laten zien dat phosphoproteomics studies interessante en relevante kandidaateiwitten kunnen opleveren, maar dat het vervolgonderzoek van een grootschalige phosphoproteomicsstudie belangrijk is om inzicht te krijgen in de biologische functie van kandidaateiwitten, zoals hier beschreven voor MKKK7. Het onderzoek naar de functionele analyse van MKKK7 heeft belangrijke nieuwe inzichten opgeleverd over de antagonistische interacties tussen twee belangrijke signaaltransductieroutes die enerzijds betrokken zijn bij pathogeen-geïnduceerde immuunresponsen en anderzijds bij groei en ontwikkeling. Een regulatoreiwit zoals MKKK7 stelt de plant instaat op een efficiënte manier te kiezen voor groei en ontwikkeling of voor activatie van de afweerresponsen tegen microbiële ziekteverwekkers. De bevindingen kunnen mogelijk in de toekomst gebruikt worden om planten te ontwikkelen die nog beter groeien en resistenter zijn tegen bacteriële ziekteverwekkers.

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data kunnen laten zien. Paul, jij leerde mij de eerste stappen van massaspectrometrie en jouw interesse in de biologische achtergrond was uniek.

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I also worked and lived a while in Ithaca, New York and I have great memories of this time, since I met so many wonderful people in the Food Science lab. Kathryn and Martin, thanks for the opportunity to work with you, working in your lab inspired me to pursue my ambitions. My former food safety colleagues; Sharinne, David, Steven, Esther, Soraya and Marie you immediately made me feel at home in a strange country and although we are currently all living in different parts of the globe, I am happy that we still remain friends.

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CURRICULUM VITAE

It was a sunny afternoon on the 31st of July, that Sharon Mithoe was born in Paramaribo, the bustling capital of Suriname. She grew up in this beautiful South American country with a small population and stunning Amazonian rainforest. After receiving her Athenaeum degree, she had to wait a year before permission was granted to leave Suriname to study in the Netherlands. The first years in the Netherlands she studied Medical Biotechnology at the Hogeschool Rotterdam and omstreken in Delft. After receiving her HLO-degree, she started working in 1993 as a research technician; at first at the department of Clinical Oncology (LUMC) in the laboratory of Prof. Susanne Osanto and later as a technician in the quality control laboratory of Centocor B.V. (Janssen Biologics B.V.) in Leiden. In 1999 she moved to the United States, where she returned to an academic setting and worked as a senior technician in the department of Molecular Biology and Biochemistry (Rutgers University) in New Jersey with Prof. Ruth Steward. In December 2000 she made a move to Cornell University in Ithaca, New York, where she worked as a research support specialist at the department of Food Science in the group headed by Prof. Martin Wiedmann and Prof. Kathryn Boor. The project she participated in was designing DNA microarrays to study overall gene expression in *Listeria monocytogenes* and she identified novel genes regulated by Sigma factor B. Upon her return to the Netherlands in December 2002, she continued to work as a senior research technician specializing in expression profiling. Initially at the department of Molecular Biology and Microbial food safety, (University of Amsterdam), in the group of Prof. Stanley Brul, studying the responses of *Bacillus subtilis* to environmental stress. In June 2004, she accepted a senior technician position at the department of Molecular Genetics (Utrecht University), headed by Prof. Peter Weisbeek, where she worked in the DNA microarray facility and studied the role of MAPKs in transcriptional reprogramming during defense-related responses in *Arabidopsis thaliana*. Inspired by the work on MAPK signaling, Sharon applied for a personal grant (Casimir/NWO) to conduct her own PhD research project. Since June 2006 she worked under the daily supervision of Dr. Frank Menke at Utrecht University on the role of protein phosphorylation in defense-related signaling in *Arabidopsis thaliana.* The project was partly carried out in collaboration with the agro-biotech company Keygene N.V. in Wageningen, where Dr. Marcel Prins was her daily supervisor.

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