

Review

Challenges for molecular plant pathology over the next ten years

Post-translational modification of host proteins in pathogen-triggered defence signalling in plants

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SUMMARY

Microbial plant pathogens impose a continuous threat to global food production. Similar to animals, an innate immune system allows plants to recognize pathogens and swiftly activate defence. To activate a rapid response, receptor-mediated pathogen perception and subsequent downstream signalling depends on post-translational modification (PTM) of components essential for defence signalling. We discuss different types of PTMs that play a role in mounting plant immunity, which include phosphorylation, glycosylation, ubiquitination, sumoylation, nitrosylation, myristoylation, palmitoylation and glycosylphosphatidylinositol (GPI)-anchoring. PTMs are rapid, reversible, controlled and highly specific, and provide a tool to regulate protein stability, activity and localization. Here, we give an overview of PTMs that modify components essential for defence signalling at the site of signal perception, during secondary messenger production and during signalling in the cytoplasm. In addition, we discuss effectors from pathogens that suppress plant defence responses by interfering with host PTMs.

INTRODUCTION

Plants are continuously challenged by a variety of organisms, such as viruses, bacteria, fungi, oomycetes, nematodes and insects. Microbes that manage to circumvent structural barriers such as the cell wall and the cuticle are generally not able to invade a plant because of the activation of a primary defence response resulting in non-host resistance. Most of the microbes that are able to evade or suppress the primary defence response are recognized by the plant via the effector proteins that they secrete, which results in the activation of a secondary defence response that in most cases involves a hypersensitive response

(HR). Eventually, only a small subset of microbes has evolved into successful pathogens that are able to suppress and/or circumvent both the primary and the secondary defence responses of the plant (Bent and Mackey, 2007; Chisholm *et al.*, 2006; De Wit, 2007; Jones and Dangl, 2006; Nürnberger *et al.*, 2004). These pathogens cause disease, and can result in severe crop losses.

The primary and secondary defence responses of plants leading to resistance rely on the swift activation of signal transduction cascades, whereby cellular changes caused by the secondary defence response are generally most pronounced (Jones and Dangl, 2006). Research on the molecular aspects of recognition and subsequent defence signalling was initiated by the proposition of the gene-for-gene hypothesis by Flor (1942). Since then, many sophisticated pathogen recognition mechanisms have been discovered that initiate highly complex signalling cascades, eventually leading to host genotype-specific resistance. Thus far, the main focus of molecular phytopathologists has been the identification and functional analysis of resistance (R) proteins and their cognate pathogen effectors, the so-called race-specific elicitors (Bent and Mackey, 2007; Takken and Tameling, 2007). In addition, transcriptional changes that occur upon pathogen recognition have been extensively studied by microarray and cDNA-AFLP experiments (Eulgem, 2005; Wise *et al.*, 2007), and the role of individual genes in resistance has been studied by transient/stable knockdown and knockout studies (Baulcombe, 1999; Burch-Smith *et al.*, 2004; Glazebrook *et al.*, 1997).

Initial plant defence responses occur extremely fast upon recognition of a pathogen (Laxalt and Munnik, 2002; Nürnberger and Scheel, 2001; Wojtaszek, 1997), which implies the involvement of post-translational modifications (PTMs) of pre-existing proteins in signal transduction cascades. A definite role for PTMs in defence signal transduction became apparent with the discovery of protein phosphorylation events in parsley cells upon elicitor treatment (Dietrich *et al.*, 1990), and with the observation that activated mitogen-activated protein kinases (MAPKs), which require phosphorylation for activation, are involved in the primary resistance response of parsley to *Phytophthora sojae* (Ligterink *et al.*, 1997). Furthermore, some receptors contain kinase domains

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themselves, which enable them to phosphorylate downstream substrates (Martin *et al.*, 2003; Van Ooijen *et al.*, 2007). In recent years, the general importance of PTMs in signal transduction cascades has become clear (Thurston *et al.*, 2005; Xing *et al.*, 2002) and its relevance for successful plant defence signalling was further confirmed by reports describing direct manipulation of PTMs by pathogens in order to suppress plant immune responses (Kim *et al.*, 2005b; Mudgett, 2005; Shan *et al.*, 2007). In this review we will discuss different types of host protein PTMs that play a role in plant defence signalling. In addition, we discuss effectors from pathogens that specifically interfere with host PTMs to suppress plant defence responses, thereby underlining the importance of PTMs in defence signalling.

POST-TRANSLATIONAL MODIFICATIONS, HOW DO THEY OCCUR?

Single genes can give rise to a diversity of RNA transcripts because of gene splicing and each of these transcripts is translated into a protein that can subsequently be proteolytically processed and/or post-translationally modified. PTMs are responsible for a major increase in complexity from genome to proteome. For example, the human genome, containing approximately 30 000 open reading frames, is predicted to give rise to approximately 1.8 million different protein species (Jensen, 2004; Kersten *et al.*, 2006). PTMs are involved in protein regulation and are therefore often reversible, rapid, controlled and highly specific but they usually affect only a small percentage of the total pool of a specific protein (Johnson, 2004). Furthermore, PTMs are catalysed by specific enzymes that in turn are often also regulated by PTMs (Peck, 2006). Currently, more than 300 types of PTMs have been described (Jensen, 2004), but here we focus on the major PTMs that have been implicated in defence signalling.

Phosphorylation

Phosphorylation is the most predominant covalent modification of proteins and implies the reversible attachment of a phosphate group to an amino acid residue. Phosphorylation has been described to play a major role in defence signalling cascades (de la Fuente van Bentem and Hirt, 2007; Peck, 2003; Thurston *et al.*, 2005; Xing *et al.*, 2002). Four types of phosphorylation occur, of which N-, S- and acyl-phosphorylation are very uncommon. O-phosphorylation is the most common type and is here further referred to as phosphorylation. Phosphorylation mainly occurs on the hydroxyl group of hydroxyamino acids such as serine, threonine and tyrosine but can also occur on unusual residues such as hydroxy-proline (Reinders and Sickmann, 2005). Phosphorylation is executed by protein kinases that transfer a phosphoryl (PO₃) group from ATP to the hydroxyl group in the polar rest (R-) group of the amino acid residue, resulting in a phosphoester (R-O-PO₃)

bond. Dephosphorylation occurs by protein phosphatases that hydrolyse the phosphoester bond, thereby releasing the phosphoryl group and restoring the hydroxyamino acid into its unphosphorylated state (Sickmann and Meyer, 2001). Generally, only a small percentage of the total pool of a certain protein in the cell is phosphorylated and a transient change of only a few percent can be sufficient to activate signalling. The opposite activity of kinases and phosphatases balances phosphorylation-based signalling cascades, rendering them highly dynamic (Reinders and Sickmann, 2005).

Ubiquitination

Another highly dynamic PTM that is implicated in defence signalling is ubiquitination. Ubiquitination refers to a three-step enzymatic cascade to covalently attach a small conserved polypeptide, ubiquitin, to a protein. First, the C-terminal glycine of ubiquitin, which is matured by deubiquitination enzymes (DUBs), forms together with the thiol group (SH) of a cysteine in the active site of the ubiquitin-activating enzyme (E1), a thioester (R_{E1}-S-CO-R_{Ub}). Subsequently, the activated ubiquitin is transferred to a cysteine residue of the ubiquitin-conjugating enzyme (E2). Finally, the ubiquitin-ligase protein (E3), which interacts with the ubiquitinated E2 enzyme, initiates attachment of the ubiquitin moiety to the target protein by an isopeptide bond between the C-terminal glycine of ubiquitin and the ε-amino group of a lysine residue of the target protein (Vierstra, 2003). The target protein often requires phosphorylation prior to binding to the E3 complex. To form a polyubiquitinated protein, these three steps are repeated so that each new ubiquitin moiety is attached to a lysine residue of the previous ubiquitin moiety. Polyubiquitination can lead to lysine (K) 48- and K63-linked chains, depending on which lysine in the ubiquitin moiety is targeted for ubiquitination, and on the E2 conjugating enzyme. Proteins modified with a K48-chain are normally targeted to the 26S proteasome for degradation, whereas K63-chains are involved in endocytosis of the protein, its activation or modification of its activity (Angot *et al.*, 2007). Some proteins are only monoubiquitinated and this may also trigger a change in the localization and/or activity of the protein (Haglund *et al.*, 2003). Eventually, DUBs are capable of removing the covalently bound ubiquitin moieties thereby changing the fate of the protein, but they also recycle ubiquitin moieties from ubiquitinated proteins processed by the proteasome (Kerscher *et al.*, 2006; Vierstra, 2003).

Sumoylation

Similar to ubiquitination, proteins can be decorated with a small ubiquitin-related modifier (SUMO) moiety during defence signalling (Miura *et al.*, 2007; Novatchkova *et al.*, 2004). Sumoylation has been reported in cell-cycle activity, DNA repair, nuclear

localization, enzymatic activity and stability of proteins and in the modulation of transcription factor activity (Miura *et al.*, 2007). Similar to ubiquitin, SUMO is processed to expose its C-terminal glycine that is subsequently attached to a lysine residue of a target protein, via conjugation machinery similar to that for ubiquitination. However, sumoylation differs from ubiquitination as it has only one universal E2-conjugating enzyme that does not always require an E3-ligase to transfer SUMO to the targeted protein. Furthermore, a weak consensus motif for sumoylation has been identified in target proteins and normally only mono-sumoylation occurs although poly-sumoylation has been reported. Finally, the cysteine proteases required for SUMO maturation and desumoylation belong to a distinct family of ubiquitin-like protein proteases (ULPs) (Chosed *et al.*, 2006).

S-nitrosylation

S-nitrosylation of proteins is another mechanism to regulate cellular processes and although not very well described, this modification is regarded as influential as protein phosphorylation (Lindermayr *et al.*, 2006). Protein S-nitrosylation occurs on cysteine residues, mainly via two mechanisms. Proteins can either become S-nitrosylated via an oxygen-dependent reaction where nitrosonium (NO^+) reacts with a thiolate group (R-S^-) of the cysteine in the protein, or nitric oxide (NO) can be transferred from a nitrosothiol (SNO) to the thiol group (SH) of the cysteine (trans-nitrosylation). SNOs consist of small molecules, like glutathione, with a thiol group (GSH), that react with NO resulting in S-nitrosoglutathione (GSNO), and are suggested to be the NO reservoirs and NO donors in the cell (Lindermayr *et al.*, 2006). Although reports on S-nitrosylation during plant–pathogen interactions are rare, the production of NO and its signalling function during plant–pathogen interactions are well described (Romero-Puertas *et al.*, 2004). The presence of GSNO reductase activity in plants, which releases NO from GSNO, indicates that the formation of SNOs could play an important role in NO signalling (Lindermayr *et al.*, 2005).

Glycosylation

Covalent linkage of an oligosaccharide side chain to a protein is referred to as protein glycosylation. The two most predominant types are N-glycosylation and O-glycosylation. Here, we only consider N-glycosylation, which can affect the asparagine residue in the sequence motif asparagine–X–serine/threonine (X can be any amino acid except proline) and which refers to the oligosaccharide side chain attachment to the asparagine residue. N-glycosylation starts co-translationally at the endoplasmic reticulum (ER) by the transfer of an oligosaccharide precursor, $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, onto the amide nitrogen of the asparagine residue. Subsequently, the oligosaccharide matures by the removal

of glucose and mannose residues or by the attachment of new sugar residues to generate glycans and complex-type glycans (Saint-Jore-Dupas *et al.*, 2007). Glycosylation occurs quite frequently and can affect the biological activity and the function of proteins, and has been reported to occur also on resistance proteins (Van der Hoorn *et al.*, 2005).

N-myristoylation and S-palmitoylation

Next to the attachment of sugars to proteins, proteins can also be modified co-translationally (N-myristoylation) or post-translationally (S-palmitoylation) with fatty acids. N-myristoylation, also referred to as myristoylation, is the modification of a protein with myristate, a hydrophobic 14-carbon fatty acid. Catalysed by N-myristoyltransferase, myristate is in general covalently and irreversibly attached through amide linkage to the N-terminal glycine exposed after removal of the initial methionine residue of the target protein by aminopeptidases. Myristoylation targets proteins to a membrane and thereby promotes interactions between these proteins and membrane-associated protein complexes (Farazi *et al.*, 2001). Protein myristoylation plays an important role in defence signalling in tomato against *Pseudomonas syringae* (Andriotis and Rathjen, 2006). S-palmitoylation, also referred to as S-acylation, is the thioesterification of palmitate (a 16-carbon fatty acid) to a cysteine residue in a protein. S-palmitoylation is catalyzed by palmitoyl acyltransferases (PAT) or occurs via a spontaneous autoacylation in the presence of long-chain acyl-coenzyme As (CoAs) and lipid vesicles. S-palmitoylation supports initial plasma-membrane binding of proteins (Smotrys and Linder, 2004), including proteins required for the perception of pathogen elicitors, and might play a role in protein trafficking (Kim *et al.*, 2005a).

GPI-anchoring

GPI-anchoring implies the attachment of a glycosylphosphatidylinositol (GPI) to anchor cell-surface proteins to the plasma membrane, where they can play a role in elicitor perception. GPI is synthesized at the ER via the sequential linkage of sugars and other components to phosphatidylinositol (PI). GPI transamidases recognize and cleave the C-terminal GPI attachment signal peptide of the target and mediate attachment to the GPI anchor. The GPI-anchored protein is subsequently secreted via the Golgi apparatus and attached to the plasma membrane (Maeda *et al.*, 2006).

PTMS OF HOST PROTEINS INVOLVED IN SIGNAL PERCEPTION

Pathogen recognition is mediated by a group of protein receptors which can be divided in a few major classes. Two classes account

for the receptor-like proteins (RLPs) and the receptor-like kinases (RLKs) that are localized in the plasma membrane and contain extracellular leucine-rich repeats (LRRs). The RLPs lack a cytoplasmic signalling domain, while RLKs have a cytoplasmic kinase domain. Two other classes are formed by receptors that are cytoplasmically localized and that contain a nucleotide-binding (NB) site and LRRs. One class is referred to as TIR-NB-LRRs as these NB-LRRs contain an N-terminal domain similar to the *Drosophila* Toll receptor and the interleukin 1 receptor (TIR). The other class is referred to as CC-NB-LRRs, as N-terminal domain structures in which frequently coiled-coil (CC) motifs are predicted are found in addition to the NB-LRR domains (Martin *et al.*, 2003; Van Ooijen *et al.*, 2007). It has become clear that the primary (non-host) defence response elicited by microbe-associated molecular patterns (MAMPs) and the secondary (host genotype-specific) defence response induced by race-specific elicitors are in fact mediated by very similar receptors (Gómez-Gómez and Boller, 2000; Zipfel *et al.*, 2006). These receptors are today referred to as pattern recognition receptors (PRRs) and R proteins, respectively (Bent and Mackey, 2007; Jones and Dangl, 2006).

Signal perception by RLKs

The best studied model system in Arabidopsis for primary defence signalling is the perception of bacterial flagellin, or its 22-amino-acid conserved epitope, flg22, by the membrane-bound PRR FLS2. FLS2 is an RLK and autophosphorylation of its kinase domain seems to be required for binding of flg22 and might affect the stability of the FLS2-flg22 complex (Gómez-Gómez *et al.*, 2001). Mutation of four, probably not autophosphorylated, phosphorylation sites in the C-terminal region of the protein did not affect flg22 binding but abolished or reduced downstream signalling. Mutation of one of these sites also significantly reduced FLS2 internalization by endocytosis (Robatzek *et al.*, 2006). FLS2 endocytosis might be triggered by ubiquitination as the required conserved (PEST) motif is present in the cytoplasmic region of the FLS2 protein, and FLS2 endocytosis is followed by its degradation (Fig. 1; Robatzek *et al.*, 2006). It has been found recently that the FLS2 receptor specifically binds to one of the somatic embryogenesis receptor kinases, SERK3, also referred to as BRI1-associated receptor kinase 1 (BAK1), in a ligand-dependent manner (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). Upon perception of brassinosteroids (BRs), which are plant steroid hormones, BAK1 forms a heterodimer with the plasma membrane receptor kinase BRI1 (BRASSINOSTEROID-INSENSITIVE 1). Both BAK1 and BRI1 display BR-dependent phosphorylation (Wang *et al.*, 2005), which enhances the interaction and complex formation between the two proteins that are subsequently internalized via endocytosis (reviewed by Karlova and de Vries, 2006). Possibly, the ligand-dependent FLS2-BAK1 complex formed *in vivo* is internalized in a similar way to the BRI1-BAK1 complex

(Fig. 1; Chinchilla *et al.*, 2007). The kinase-associated protein phosphatase (KAPP) might negatively regulate FLS2 signalling as it binds and dephosphorylates FLS2 (Gómez-Gómez *et al.*, 2001). Recently, a highly homologous receptor that recognizes an 18-amino-acid fragment of the bacterial elongation factor Tu (EF-Tu) was identified (Zipfel *et al.*, 2006). Just like FLS2, this EF-Tu receptor (EFR) requires BAK1 for downstream signalling and upon stimulation both PRRs induce the transcription of a similar set of genes, including a large amount of additional RLKs. Furthermore, they induce a common set of responses including downstream MAPK activation and extracellular alkalization (Chinchilla *et al.*, 2007; Zipfel *et al.*, 2006). Phosphorylation of the EFR receptor itself has not yet been reported, but the homology to the FLS2 signalling cascade suggests a role for EFR-mediated phosphorylation upon EF-Tu perception (reviewed by Nürnberger and Kemmerling, 2006). In addition, an RLK referred to as RPG1 confers resistance of barley to *Puccinia graminis* f. sp. *tritici*. RPG1 contains two tandem kinase domains of which only the C-terminal domain is functional and displays autophosphorylation required for resistance. In accordance with FLS2 signalling, RPG1 appears to be degraded in a proteasome-dependent way upon inoculation with an avirulent strain, which implies that RPG1 becomes ubiquitinated (Nirmala *et al.*, 2006, 2007). Furthermore, the rice RLK Xa21, which mediates recognition of the effector AvrXa21 from *Xanthomonas oryzae* pv. *oryzae* (Song *et al.*, 1995), has a kinase domain that autophosphorylates on several serine and threonine residues and that stabilizes the protein and probably protects it from proteolytic cleavage (Liu *et al.*, 2002; Xu *et al.*, 2006). Xa21 phosphorylates the Xa21-binding protein 3 (XB3) that binds *in vivo* to the receptor and which is required for its accumulation. XB3 is a RING finger-containing protein that can function as an E3 ubiquitin ligase and it is hypothesized that XB3 is phosphorylated by Xa21 upon pathogen recognition. XB3 subsequently ubiquitinates a downstream component, which could be a negative regulator of defence signalling that is targeted for degradation (Fig. 1; Wang *et al.*, 2006). However, referring back to FLS2, XB3 might also mediate Xa21 ubiquitination and degradation. It is tempting to speculate that RLK-mediated signalling is initiated by phosphorylation and formation of a ligand-dependent protein complex that internalizes and is subsequently degraded in a proteasome-dependent manner.

Signal perception by RLPs

RLPs, which lack a kinase domain and thus lack autophosphorylation, are represented by, for example, the so-called Cf proteins of tomato plants mediate resistance to *Cladosporium fulvum* (Rivas and Thomas, 2005). One of the family members is Cf-9, which is highly glycosylated, a feature required for its stability and for a full Cf-9-mediated HR. Probably, Cf-9 *N*-glycosylation is required for a stable structural conformation and/or interactions

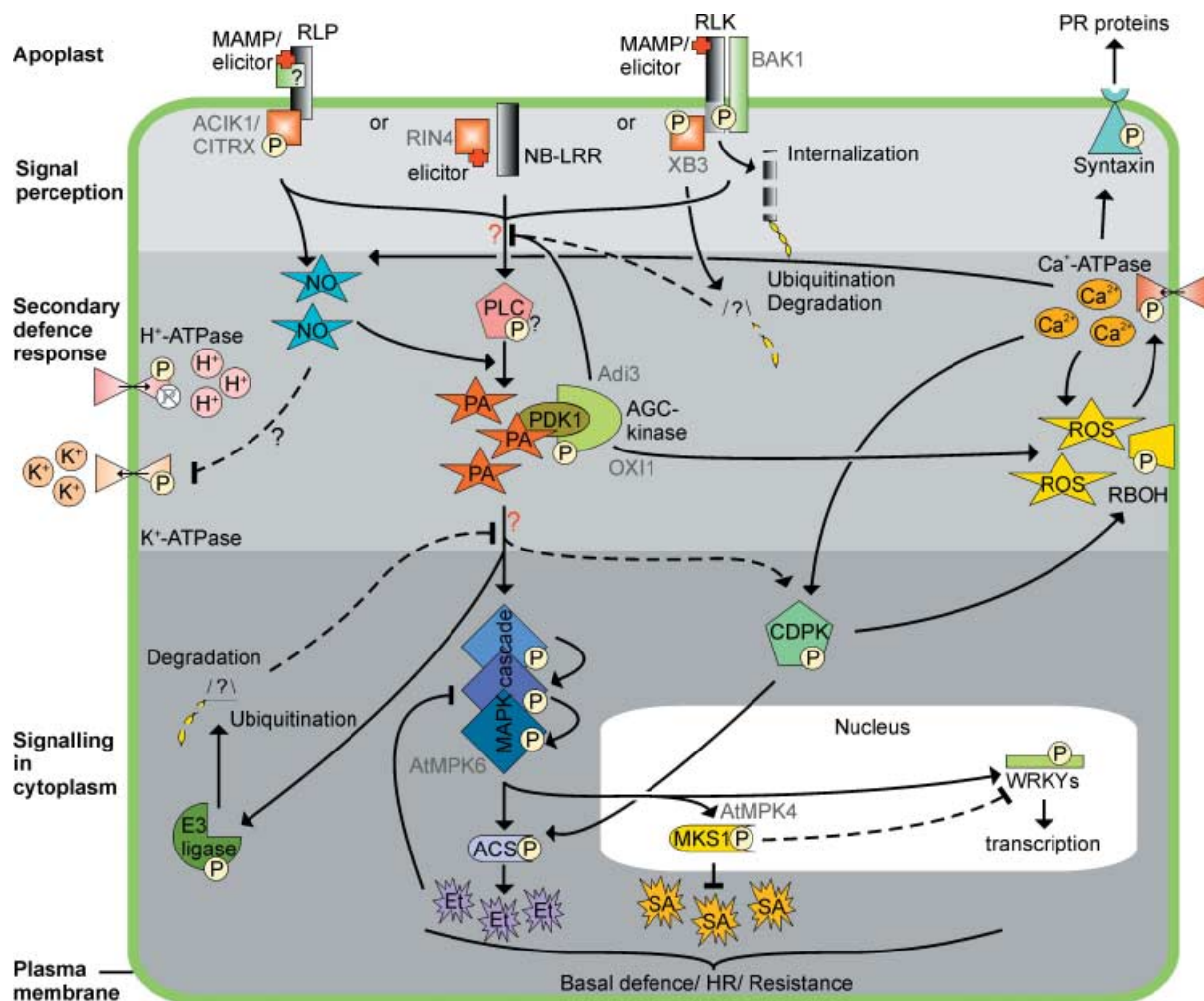


Fig. 1 Defence-related signal transduction cascades that depend on post-translational modifications. Receptors mediate recognition of microbe-associated molecular patterns (MAMPs) and race-specific elicitors (elicitor), but they require additional proteins for their function. Proteins with nucleotide-binding and leucine-rich repeat domains (NB-LRR) recognize their cognate elicitors intracellularly, while receptor-like proteins (RLP) and receptor-like kinases (RLK) are probably activated outside the cell. RLPs require additional proteins that bind the cytoplasmically localized part of the protein to mediate downstream signalling, while RLKs require their kinase domain to autophosphorylate and form complexes with additional proteins. RLKs might become ubiquitinated, after which they are internalized and targeted for proteasome-mediated degradation. Signalling downstream from the receptor eventually leads to the formation of secondary messengers such as phosphatidic acid (PA), possibly via phospholipase C (PLC) phosphorylation, and nitric oxide (NO). The concentrations of ions such as H⁺, K⁺ and Ca²⁺ are controlled by (de)phosphorylation of the respective ATPase while the production of ROS is stimulated upon phosphorylation of the NADPH oxidases (RBOH). The secondary messengers also mediate phosphorylation of proteins such as calcium-dependent protein kinases (CDPK), or syntaxins, which might promote the release of pathogenesis-related (PR) proteins into the apoplast. Mitogen-activated protein kinase (MAPK) cascades are activated by phosphorylation of the individual components, which eventually leads to the phosphorylation of, amongst others WRKY transcription factors, 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and MAP kinase substrate 1 (MKS1), which influence the production of ethylene (Et) and salicylic acid (SA), respectively. Also, E3-ligases are activated, which might result in the ubiquitination and subsequent degradation of negative regulators of the signalling cascades, thereby providing a positive feedback loop. In addition, negative feedback loops are required to prevent an uncontrolled hypersensitive response (HR). For example, MAPK (MPK)-mediated ethylene production negatively regulates MAPK activation. The secondary messengers influence each other and fine-tune the downstream signal while proteins modified by secondary messengers might inhibit receptor-mediated signals. Eventually, a balanced signal will lead to increased (basal) resistance and possibly a HR. Phosphorylation states as presented in this figure represent the active state of the protein. Protein names indicated in grey might be specific for a particular plant–pathogen interaction. ACIK1, Avr9/Cf-9-induced kinase 1; CITRX, Cf-9-interacting thioredoxin; RIN4, RPM1-interacting protein 4; BAK1, BRASSINOSTEROID-INSENSITIVE 1; XB3, Xa21-binding protein 3; Adi3, AvrPto-dependent Pto-interacting protein 3; PDK1, 3-phosphoinositide-dependent protein kinase-1; AGC-kinase, protein kinase A, G and C family; OXI1, oxidative signal-inducible 1.

with the cell wall (Piedras *et al.*, 2000; Van der Hoorn *et al.*, 2005). Cf-9 has no signalling domain but the cytoplasmic C-terminus interacts with a thioredoxin (CITRX; for Cf-9-interacting thioredoxin) that accelerates the Cf-9/Avr9-induced HR upon transcriptional knockdown by virus-induced gene silencing (VIGS) (Rivas *et al.*, 2004). In addition, the Avr9/Cf-9-induced kinase 1 (ACIK1), which encodes a cytoplasmic serine/threonine kinase, compromises the Cf-9/Avr9- and Cf-4/Avr4-induced HR and resistance upon VIGS (Durrant *et al.*, 2000; Rowland *et al.*, 2005). Interestingly, ACIK1 binds and phosphorylates CITRX and binds the C-terminus of Cf-9 with CITRX as an adaptor protein, thereby forming a complex that can mediate downstream signalling (Nekrasov *et al.*, 2006). However, it remains difficult to understand how the downstream signalling from Cf-9 actually takes place given that ACIK1 is a positive regulator and CITRX a negative regulator of Cf-9/Avr9-induced defence signalling, and the catalytic domains are not required for the interaction between the different proteins (Nekrasov *et al.*, 2006). We hypothesize that Cf-9, CITRX and ACIK1 form a complex under normal conditions in unchallenged plants. Upon elicitation by Avr9, ACIK1 phosphorylates CITRX, which destabilizes the complex and releases CITRX and ACIK1 into the cytoplasm where they can activate downstream signalling components resulting in a balanced defence response (Fig. 1). In addition to race-specific elicitor recognition, RLPs also mediate MAMP-induced defence responses. The MAMP xylanase from *Trichoderma viride* triggers signalling through the ethylene-inducing xylanase (EIX) PRR, which is an RLP (Ron and Avni, 2004). Chitin, a major component from fungal cell walls, is a MAMP that triggers signalling by the chitin oligosaccharide elicitor-binding protein CEBiP, which is an RLP that is highly glycosylated, just like Cf-9 (Kaku *et al.*, 2006). However, for these PRRs it remains to be elucidated how the perceived signal is transferred further downstream to the cytoplasm.

Signal perception by NB-LRRs

Resistance to *Pseudomonas* species is in most cases conferred by NB-LRRs. The interaction between tomato and *Pseudomonas syringae* pv. *tomato* (Pst) is a well-studied model system. Resistance to Pst expressing the elicitor genes *AvrPto* and/or *AvrPtoB* requires the *Pto* and the *Prf* genes (Kim *et al.*, 2002; Salmeron *et al.*, 1994). *Pto* encodes a serine/threonine protein kinase (Loh and Martin, 1995) and, originally, *Pto* was reported as the AvrPto-matching R protein (Martin *et al.*, 1993). However, further analysis revealed *Prf* as a CC-NB-LRR protein, which is capable of signalling in the absence of *Pto*, while *Pto* is incapable of signalling in the absence of *Prf* (Salmeron *et al.*, 1996). It was also shown that *Prf* and *Pto* interact *in vivo* and that *Prf* accumulates to higher levels in the presence of *Pto* (Mucyn *et al.*, 2006). Therefore, *Prf* is now classified as the R protein that activates downstream signalling (Van Ooijen *et al.*, 2007). Still, *Pto* plays

an important role in AvrPto and AvrPtoB perception as *Pto* specifically binds both elicitors and several other *Pto*-interacting (*Pti*) proteins (Kim *et al.*, 2002; Sessa *et al.*, 2000b; Tang *et al.*, 1996). *Pti1* represents a serine/threonine kinase which is phosphorylated by the *Pto* kinase, and this phosphorylation is required for *Pto*/*Pti1* interaction (Sessa *et al.*, 2000a). *In vitro*, *Pto* autophosphorylates at eight sites, three of which are required for HR development and AvrPto binding, and one is only required for HR development, indicating that *Pto* kinase activity is required for the AvrPto/*Prf*-dependent HR elicitation (reviewed by Pedley and Martin, 2003). Further research revealed two additional phosphorylation sites in the activation loop of *Pto* required for AvrPto binding. Substitution of these residues by aspartic acid (D), which mimics the negative charge introduced by phosphorylation, resulted in a *Prf*-dependent and AvrPto-independent HR in tomato (Rathjen *et al.*, 1999). To complicate *Pto*-mediated AvrPto perception further, *Pto* was also found to be myristoylated at the N-terminus, which negatively regulates its kinase activity (Andriotis and Rathjen, 2006). A model summarizing these results has been proposed: *Pto* is myristoylated to suppress its kinase activity and to be targeted to a cellular membrane, most likely the plasma membrane, where it binds to *Prf*. AvrPto targets the complex and causes displacement of the myristoylated N terminus of *Pto*, which results in derepression of the kinase domain, *Pto* phosphorylation and activation, and subsequent signalling via *Prf* (Andriotis and Rathjen, 2006; Balmuth and Rathjen, 2007). Furthermore, AvrPto and phosphorylated *Pto* form a complex with AvrPto-dependent *Pto*-interacting protein 3 (*Adi3*). *Adi3* is a member of the AGC family of protein kinases (protein kinase A, G and C family) and negatively regulates *Pto*-AvrPto-induced host cell death when phosphorylated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) or *Pto*. In contrast to the AvrPto-dependent *Pto*/*Adi3* interaction, *Adi3* phosphorylation by *Pto* is independent of AvrPto and is not required for *Pto*/AvrPto/*Adi3* complex formation. Therefore, it is hypothesized that *Adi3*-mediated negative regulation is released when bound to the *Pto*-AvrPto complex (Devarenne *et al.*, 2006). Possibly, phosphorylated *Adi3* negatively regulates elicitor-independent *Pto* signalling under normal conditions to avoid activation of defence responses. Upon elicitation, *Adi3* is dephosphorylated and binds to *Pto*, which leads to *Pto*-mediated signalling. As described in other defence signalling cascades, secondary messengers such as phosphatidic acid might be produced (see below) that bind and possibly activate PDK1 (Testerink *et al.*, 2004). PDK1 might subsequently phosphorylate *Adi3* to negatively regulate *Pto* signalling again, thereby forming a negative feedback loop (Fig. 1).

Interactions between *Pseudomonas syringae* and Arabidopsis are also intensively studied and several intracellular NB-LRRs have been described to mediate recognition of elicitors from different *P. syringae* strains (Nimchuk *et al.*, 2003). The R proteins RPS2 and RPS5 provide resistance to *P. syringae* pathovars

expressing AvrRpt2 or AvrPphB, respectively, whereas RPM1 provides resistance to *P. syringae* pathovars expressing AvrRpm1 or AvrB (reviewed by Belkhadir *et al.*, 2004b). In a yeast two-hybrid screen, two RPM1-interacting proteins (RINs), RIN2 and RIN3, were identified which represent RING-finger ubiquitin E3 ligases and which also weakly interact with RPS2. These RINs seem to enhance the RPM1- and RPS2-mediated HR; however, they do not restrict bacterial growth in the plant. Although RIN2 and RIN3 encode proteins that show E3 ligase activity *in vitro*, a target protein that might serve as a negative regulator of the HR and is degraded remains to be identified (Kawasaki *et al.*, 2005). Another protein that physically interacts with RPM1 and RPS2 is RIN4, a protein that negatively regulates RPM1- and RPS2-mediated resistance (Belkhadir *et al.*, 2004a). RIN4 is C-terminally palmitoylated, which is required for RIN4 localization to the plasma membrane and its functioning (Belkhadir *et al.*, 2004a; Day *et al.*, 2005; Kim *et al.*, 2005a). It is hypothesized that RIN4 is bound to RPM1 and RPS2 under normal conditions to regulate defence signalling negatively and that RIN4 is released from the complex upon R protein triggering (Fig. 1). In addition, defence signalling by RPM1 or RPS2 requires the non-race-specific disease resistance 1 (NDR1) protein, which is glycosylated and C-terminally processed. NDR1 is thought to undergo GPI modification at its processed C-terminus and this GPI-anchor places the protein on the outer surface of the plasma membrane with a short part of the N-terminus in the cytoplasm, where it binds the C-terminal half of RIN4 (Coppinger *et al.*, 2004; Day *et al.*, 2006). Upon inoculation with an AvrRpt2-producing Pst strain, RIN4 is cleaved by the cysteine protease activity of the AvrRpt2 effector, after which the negative regulation of RPS2 by RIN4 is released (Fig. 1; Takemoto and Jones, 2005). A C-terminal membrane-embedded RIN4 fragment is not degraded after cleavage and positively regulates RPS2-mediated signalling by its interaction with NDR1 (Day *et al.*, 2006). Probably, RIN4-mediated RPM1 activation by AvrRpm1/AvrB elicitation occurs via a different mechanism, as RIN4 degradation abolishes RPM1 signalling, and RPM1 activation depends on RIN4 phosphorylation (Kim *et al.*, 2005a,b). In addition to RPM1 and RPS2, also RPS5-mediated resistance depends on NDR1 but RPS5 does not require RIN4 (Coppinger *et al.*, 2004). Instead, RPS5-mediated resistance to *P. syringae* depends on a serine/threonine protein kinase PBS1 that binds to RPS5 in unchallenged plants (Ade *et al.*, 2007; Swiderski and Innes, 2001). Similar to AvrRpt2-mediated cleavage of RIN4 and the subsequent activation of RPS2, the cysteine protease AvrPphB cleaves PBS1, which activates RPS5. PBS1 requires a functional kinase domain that is probably involved in autophosphorylation; however, neither the phosphorylation nor the elimination of PBS1 is sufficient to activate RPS5. Therefore, it is hypothesized that a phosphorylated cleavage product of PBS1 is required for RPS5 activation (Shao *et al.*, 2003).

PTMS OF PLASMA MEMBRANE-LOCALIZED HOST PROTEINS INVOLVED IN DOWNSTREAM SIGNALLING

In addition to the above described complexes that are at least partially localized and/or bound to the plasma membrane, several other post-translationally modified membrane-localized proteins exist that are not directly involved in signal perception but play a role in downstream responses.

Transport of secondary messengers over the plasma membrane by ATPases

Secondary messengers are transported over membranes by pumps that are driven by the hydrolysis of ATP and are referred to as ATPases. An important subclass of ATPases is formed by the H⁺-ATPases that mediate the generation of electrochemical gradients across the plasma membrane, which is the energy source for most transport proteins (Palmgren, 2001). H⁺-ATPases require phosphorylation on a threonine residue in the N-terminus for their activity and are inactivated by dephosphorylation of this site. However, a plasma membrane H⁺-ATPase from Arabidopsis was also inactivated by phosphorylation on a serine residue by the PKS5 serine/threonine protein kinase. This phosphorylation event prevents interaction with a 14-3-3 protein and therefore inhibits the activity of the H⁺-ATPase (Fig. 1; Fuglsang *et al.*, 2007). Furthermore, plasma membrane-bound H⁺-ATPases are dephosphorylated upon recognition of the Avr5 elicitor of *C. fulvum* by Cf-5 tomato suspension cells (Vera-Estrella *et al.*, 1994). In addition to H⁺-ATPases, Ca²⁺-ATPases also seem to be regulated via phosphorylation. For example, in closing *Vicia* guard cells, Ca²⁺-ATPases become phosphorylated, which enhances Ca²⁺ import in the cell (Köhler and Blatt, 2002). Furthermore, elicitation of the plasma membrane of Cf-5 tomato protoplasts with the Avr5 elicitor activates a Ca²⁺-ATPase by G-protein-dependent phosphorylation (Fig. 1; Gelli *et al.*, 1997). Also, K⁺ channel activity seems to depend on phosphorylation. The stimulation of the K⁺ outward channels and the suppression of the K⁺ inward channels upon elicitation of transgenic Cf-9-expressing *Nicotiana tabacum* cells with Avr9 is completely blocked by broad-range protein kinase inhibitors (Blatt *et al.*, 1999). K⁺ channels might also be nitrosylated as NO blocks outward K⁺ channels in guard cells (Fig. 1; Sokolovski and Blatt, 2004).

Syntaxins and other membrane-bound proteins in defence signalling

To identify plasma membrane-bound proteins in Arabidopsis that are (de)phosphorylated upon defence signalling, ³²P pulse-labelled suspension-cultured cells were elicited with flg22 and plasma membrane proteins were analysed by two-dimensional

gel electrophoresis (Nühse *et al.*, 2003). This revealed several differentially phosphorylated proteins such as the syntaxin AtSyp122. Syntaxins are part of the SNARE complex and play a central role in exocytosis as they mediate vesicle fusion to the plasma membrane (Fasshauer, 2003). Phosphorylation of AtSyp122 is Ca^{2+} -dependent, which leads to the hypothesis that a Ca^{2+} influx stimulates exocytosis of defence proteins and other compounds via syntaxins (Fig. 1). In agreement with this hypothesis, the same phosphoproteomics screen revealed a second syntaxin, AtSyp132, of which the *N. benthamiana* orthologue, NbSyp132, contributes to the exocytosis of pathogenesis-related (PR) proteins into the apoplast upon Pto/AvrPto-induced defence signalling (Kalde *et al.*, 2007). Furthermore, NbSyp132 contributes to basal- and salicylate-associated defence against bacterial pathogens in plants (Kalde *et al.*, 2007). Another plasma membrane-localized syntaxin, Syp121 or PEN1, is required for resistance to powdery mildew in barley but does not play a role in Pto-mediated resistance to Pst (Fig. 1; Collins *et al.*, 2003; Kalde *et al.*, 2007). The orthologue NtSyp121 is phosphorylated upon Cf-9/Avr9-activated signalling, which appeared to be specific as this syntaxin is not phosphorylated upon elicitation with flg22 (Heese *et al.*, 2005).

Recent technical advances in phosphoproteomics now enable phosphopeptide or phosphoprotein purification and their immediate analysis by mass spectrometry. A non-quantitative analysis of phosphorylated plasma membrane-bound proteins from flg22-elicited Arabidopsis cells revealed over 300 phosphorylation sites although it remains unclear to what extent these phosphorylation sites play a role during signalling cascades (Nühse *et al.*, 2004). Recently, quantitative phosphoproteome studies of flg22- or xylanase-treated Arabidopsis cells revealed several differentially phosphorylated proteins. Some of these proteins, such as calcium-dependent protein kinases (CDPKs) and ATPases, have already been described to be regulated by phosphorylation, but for other proteins like auxin efflux carriers and respiratory burst oxidase protein D, phosphorylation-mediated regulation is novel (Benschop *et al.*, 2007; Nühse *et al.*, 2007). Further functional analysis of the identified phosphoproteins will reveal new insights into defence-related signalling cascades.

PTMS LEADING TO THE FORMATION OF HOST SECONDARY MESSENGERS

When a microbe is recognized by the plant, defence signalling cascades are activated. Thus far, it is unclear how signals are transferred from the receptor to one or more downstream pathways such as the MAPK pathway. Studies using suspension-cultured cells indicate that in intact plants, secondary messengers are produced upon elicitation and they are thought to play a role in amplifying and transferring the signal downstream into the signalling cascade (Laxalt and Munnik, 2002).

NO signalling

Elicitation of tomato cells with xylanase results in the production of the secondary messenger NO (Fig. 1; Laxalt *et al.*, 2007). In Arabidopsis, NO is synthesized by the NO synthase enzyme, AtNOS1 (Guo *et al.*, 2003), or results from the reduction of nitrate by nitrate reductase (NR) (Romero-Puertas *et al.*, 2004). However, other mechanisms to generate NO are also likely to exist (Neill *et al.*, 2007). To transfer a signal, the highly reactive NO molecules can modify a variety of target proteins by *S*-nitrosylation. An extensive study in Arabidopsis led to the identification of many proteins that can be modified by *S*-nitrosylation, of which some proteins such as superoxide dismutases and Hsp90 have been reported in defence signalling as well (Lindermayr *et al.*, 2005).

PA signalling

Another secondary messenger is the phospholipid-derived molecule phosphatidic acid (PA), which is produced upon signal perception via the phospholipase C or D (PLC/PLD) pathway (Bargmann and Munnik, 2006; Laxalt and Munnik, 2002; Testerink and Munnik, 2005). Most elicitors reported to induce PA production stimulate PLC-mediated formation of PA via the phosphorylation of the intermediate diacylglycerol (DAG) by DAG kinase (DGK), although some elicitors also activate the PLD pathway (Andersson *et al.*, 2006; De Jong *et al.*, 2004; Van der Luit *et al.*, 2000). The PLCs might be activated by upstream kinases that have been activated as a result of receptor triggering, as an Arabidopsis PLC was reported to be phosphorylated upon flagellin elicitation (Fig. 1; Nühse *et al.*, 2007). In soybean, PA generated upon wounding has been shown to activate the MAPK cascade as the addition of exogenous PA to suspension-cultured cells specifically activates a MAPK (Fig. 1). PA formation in wound-induced leaves can be blocked with PLD inhibitors (Lee *et al.*, 2001). Furthermore, PA stimulates the oxidative burst upon elicitation (Andersson *et al.*, 2006; De Jong *et al.*, 2004). In xylanase-treated tomato suspension cells, PA is produced via the PLC/DGK pathway, which is activated by a xylanase-triggered NO accumulation. How NO exactly activates the PLC/DGK pathway remains unclear, although NO might act directly on PLC and/or DGK by protein *S*-nitrosylation (Fig. 1). NO might also affect the PLC/DGK pathway indirectly via the MAPK signalling cascade, via altered Ca^{2+} levels or via a change in redox potential in the cell (Laxalt *et al.*, 2007; Lee *et al.*, 2001). In Arabidopsis, PA targets have been identified and include heat shock protein 90, serine/threonine kinases and phosphatases (Testerink *et al.*, 2004). Another target is the previously described phosphoinositide-dependent kinase PDK1 (Fig. 1, see above) (Anthony *et al.*, 2006). PDK1 interacts with the OX11 kinase (oxidative signal-inducible 1; also referred to as AGC2-1) and subsequently phosphorylates and activates OX11, which is involved in oxidative burst-mediated

signalling in *Arabidopsis* (Fig. 1; Anthony *et al.*, 2004, 2006; Rentel *et al.*, 2004). OX11, in turn, phosphorylates the serine/threonine kinase PTI1-2, which has high sequence homology to the tomato Pti1 kinase. The signalling pathway PDK1/OX11/PTI1-2 was shown to be specific for lipid signalling, whereas reactive oxygen species (ROS) and flagellin signals converge further downstream in the OX11/PTI1-2 pathway, independently of PDK1 (Anthony *et al.*, 2006). As the AGC kinase Adi3 is also phosphorylated by PDK1 (see above), we suggest that PDK1 functions as a spider in the web for transferring receptor-mediated PA signals to downstream signalling cascades via AGC kinases (Fig. 1). To balance the signalling cascade, PA signals are attenuated by PA kinase (PAK), which converts PA into the lipid DAG pyrophosphate (DGPP) (Munnik *et al.*, 1996). However, because DGPP accumulation is associated with PA-induced signalling, DGPP itself might also function as a secondary messenger. The observation that DGPP is broken down by the DGPP phosphatase (DPP) might confirm this hypothesis (reviewed by Van Schooten *et al.*, 2006).

ROS signalling

ROS are important secondary messengers responsible for the oxidative burst. Upon pathogen recognition, the plant responds with a bi-phasic production of ROS (Lamb and Dixon, 1997). ROS can be produced inside the plant cell in several organelles; however, a membrane-bound respiratory oxidative burst protein (RBOHD; an NADPH oxidase) is considered as the source of ROS upon elicitation by pathogens (Torres and Dangl, 2005). The *Arabidopsis* RBOHD protein is heavily phosphorylated at seven different amino acid residues and differentially phosphorylated at three residues upon elicitation with flg22 or xylanase (Benschop *et al.*, 2007; Nühse *et al.*, 2007). In accordance, another member from the RBOH family, RBOHB, is phosphorylated by calcium-dependent protein kinases (CDPKs) in potato, which causes a subsequent oxidative burst (Fig. 1; Kobayashi *et al.*, 2007). Upon signal-induced phosphorylation, the activated oxidase converts O₂ into O₂⁻, which subsequently forms the stable component hydrogen peroxide (H₂O₂), which is removed by catalases or peroxidases when the signal is transferred further down. Besides a signalling role, H₂O₂ also has direct antimicrobial effects, cross-links cell walls and activates transcription of defence-related genes (Lamb and Dixon, 1997).

Calcium signalling

A secondary messenger that links several defence-related processes is the ubiquitous messenger calcium (Ca²⁺) (Lecourieux *et al.*, 2006). Ca²⁺ is imported to and exported from the cell and the vacuole by Ca²⁺-ATPases that are regulated via phosphorylation, and stimulates the production of NO and ROS upon recognition

of an avirulent pathogen (Fig. 1; Delledonne, 2005). Strikingly, H₂O₂ also stimulates rapid Ca²⁺ influxes upon elicitation, which reveals a role for Ca²⁺ signalling up- and downstream of ROS (Fig. 1; Lamb and Dixon, 1997). These data imply that secondary messengers produced via pathways that rely on PTMs connect several components of the defence signalling cascades, but also influence each other to balance the downstream responses.

PTMS OF HOST PROTEINS IN THE CYTOPLASM

The MAPK cascade

A major conserved signalling cascade that is activated by a large range of biotic and abiotic stress stimuli in plants is the MAPK cascade (Fig. 1; Pedley and Martin, 2005; Zhang *et al.*, 2006). MAPK cascades consist of three functionally linked protein kinases that transfer the stress signals. A stress signal causes phosphorylation and activation of the most upstream MAPK kinase kinase (MAPKKK). Subsequently, the MAPKKK phosphorylates and activates a MAPK kinase (MAPKK), which in turn does the same with a MAPK (MPK). The MAPK then phosphorylates downstream target(s) thereby transferring the signal further downstream (Fig. 1). The *Arabidopsis* genome encodes 20 MAPKs, ten MAPKKs and 60 putative MAPKKKs and, in addition, it contains several protein phosphatases that control the cascade by dephosphorylating the MAPK cascade components (Ichimura *et al.*, 2002; Martín *et al.*, 2005). For example, the AtMPK6 protein, activated upon most stress stimuli, is controlled by the phosphatases ABI1, AP2C1, MKP1 and MKP2 in *Arabidopsis* (Lee and Ellis, 2007; Leung *et al.*, 2006; Schweighofer *et al.*, 2007; Ulm *et al.*, 2002). The AtMPK4 protein negatively regulates defence responses upon phosphorylation, which implies also that the activation of protein phosphatases can mediate the transfer of stress-related signals (Ichimura *et al.*, 2006; Suarez-Rodriguez *et al.*, 2007). It is interesting to note that most stress stimuli mainly activate AtMPK6, -3 and -4 and their orthologues in other plant species during stress-related signalling. Therefore, stress-related signalling cascades are considered to converge in the MAPK cascades, after which the signal is transferred into different downstream pathways (Pedley and Martin, 2005; Zhang *et al.*, 2006). In Cf-4 tomato, three highly homologous MAPKs, LeMPK1, -2 (both are orthologous to AtMPK6) and LeMPK3 (the AtMPK3 orthologue), are activated upon Avr4-elicitation. These LeMPKs appeared to have different phosphorylation specificities and a different role in defence signalling, suggesting that the signal can eventually be transferred to different substrates and possibly different downstream signalling cascades (Stulemeijer *et al.*, 2007). Thus far, only a few MAPK targets have been described. The AtMPK6 protein phosphorylates 1-aminocyclopropane-1-carboxylic acid synthase 6 and 2 (ACS6/2), which are key enzymes in ethylene biosynthesis, and WRKY transcription factors upon

flg22 elicitation (Fig. 1; Asai *et al.*, 2002; Liu and Zhang, 2004; Menke *et al.*, 2005). The AtMPK4 protein phosphorylates MAP kinase substrate 1 (MKS1), which negatively regulates salicylic acid-dependent resistance upon phosphorylation (Fig. 1; Andreasson *et al.*, 2005). Furthermore, AtMPK3 was recently found to phosphorylate the transcription factor VIP1, which is involved in regulating the expression of the PR1 pathogenesis-related gene (Djamei *et al.*, 2007).

CDPK-mediated signalling

CDPKs contain a calmodulin-like domain with Ca²⁺ binding sites and represent another class of kinases. In the absence of Ca²⁺, the kinase domain of CDPKs is not phosphorylated, which points to a direct regulation by Ca²⁺ (Fig. 1; Ludwig *et al.*, 2004). Tobacco NtCDPK2 was the first CDPK reported to be involved in plant defence signalling in transgenic Cf-9 tobacco upon elicitation with the Avr9 effector. NtCDPK2 is required for HR development and is activated by phosphorylation (Fig. 1). Furthermore, NtCDPK2 enhances ethylene production, which subsequently negatively regulates the MAPK signalling cascade (Fig. 1). In addition, a tomato CDPK phosphorylates tomato ACS2 (Tatsuki and Mori, 2001), the orthologue of which in Arabidopsis was shown to be phosphorylated by AtMPK6 (Liu and Zhang, 2004). This observation suggests that two kinase signalling cascades, both leading to an ethylene-dependent cell death, can cross-talk to fine-tune the final outcome (Ludwig *et al.*, 2005). Finally, the potato CDPK, StCDPK5, phosphorylates StRBOHB thereby regulating the oxidative burst (Fig. 1; Kobayashi *et al.*, 2007).

Ubiquitination in defence signalling

In recent years, several proteins with E3 ubiquitin ligase activity that play a role in defence signalling have been reported, indicating that ubiquitination is important for resistance of plants to pathogens (Fig. 1). An extensive transcriptional analysis of Cf-9 transgenic tobacco cells elicited with Avr9 revealed two genes, *ACRE189* and *ACRE276*, the encoded proteins of which possess *in vitro* E3 ligase activity and which are required for Cf-9- and Cf-4-mediated defence signalling (Durrant *et al.*, 2000; Yang *et al.*, 2006). The closest orthologue of *ACRE276* in Arabidopsis, PUB17, is also required for RPM1- and RPS4-mediated resistance to *Pseudomonas syringae* pv. *tomato* expressing the elicitors *AvrB* or *AvrRPS4*, respectively (Yang *et al.*, 2006). *ACRE74*, which encodes another tobacco E3 ligase (NtCMPG1), is also required for Cf-9/Avr9-induced signalling in addition to defence responses induced by Pto/AvrPto and the *Phytophthora infestans* elicitor Inf1 (González-Lamothe *et al.*, 2006). Furthermore, a functional tomato E3 ligase, ATL6, is transcriptionally upregulated upon elicitation with a cell-wall protein fraction from *Pythium*

oligandrum (Hondo *et al.*, 2007). In addition to E3 ubiquitin ligase activity, transient-induced gene silencing (TIGS) of the ubiquitin-encoding gene itself and subsequent complementation studies in powdery mildew-inoculated resistant barley suggest a role for K48-linked polyubiquitination in defence signalling. Although K48-linked polyubiquitination normally results in proteasome-mediated protein degradation, here the polyubiquitination event but not the subsequent degradation is required for the defence response (Dong *et al.*, 2006). Finally, the Arabidopsis E1 ubiquitin-activating enzyme UBA1 is required for defence responses induced upon recognition of the AvrRpt2 effector (Goritschnig *et al.*, 2007).

Sumoylation in defence signalling

In addition to ubiquitination, sumoylation also plays a role in defence signalling, although evidence for this remains scarce. Thus far, there are only two reports that show an increase in protein sumoylation upon exposure to abiotic stress conditions such as heat shock, H₂O₂, ethanol and the defensive compound against herbivores, canavanine (Kurepa *et al.*, 2003; Saracco *et al.*, 2007). However, overexpression of SUMO in tobacco appears to block HR development upon xylanase infiltration (Hanania *et al.*, 1999) and a SUMO E3 ligase, SIZ1, was reported to regulate salicylic acid-mediated innate immunity in Arabidopsis (Lee *et al.*, 2007). SIZ1 also appears to negatively regulate systemic-acquired resistance and the expression of PR genes. The best evidence for the importance of sumoylation in defence signalling originates from the observation that pathogen effectors interfere with the host sumoylation cascade. The *Xanthomonas campestris* effector XopD is injected into the host cell upon infection and encodes an active cysteine protease with plant-specific SUMO substrate specificity. XopD specifically desumoylates host proteins, thereby most likely interfering with the host defence signalling cascade upon infection (Hotson *et al.*, 2003). Another effector from *X. campestris*, AvrXv4, requires its protease activity to reduce the amount of SUMO-conjugated proteins in the host cell, which leads to suppression of localized cell death in inoculated plants (Roden *et al.*, 2004). The effector AvrBsT, which also possesses protease activity, requires its catalytic domain to induce cell death in *N. benthamiana* (reviewed by Hotson and Mudgett, 2004; Orth *et al.*, 2000). Additionally, some effectors seem to interact with proteins from the host sumoylation machinery. Xylanase interacts with SUMO in a yeast-two-hybrid system (Hanania *et al.*, 1999) and the replication protein RepAC1 from geminiviruses interacts with the SUMO E3 ligase SCE1 from *N. benthamiana* (Castillo *et al.*, 2004). However, the biological relevance of these observations remains to be elucidated. Yet, if sumoylation did not play any role in defence signalling, the various effectors mentioned above would not enhance virulence for the pathogen and they would probably

have been eliminated from the population during evolution to avoid recognition by resistant plants.

EFFECTORS OF PATHOGENS MODIFY PTMS IN DEFENCE SIGNALLING

It has become apparent from the information given above that plants depend on rapid PTMs in signalling cascades to defend themselves against intruding pathogens. An active defence response is triggered by the recognition of elicitors that are secreted by the invading pathogen. Therefore, the intriguing question remains as to why pathogens still secrete elicitors that induce avirulence. Increasing evidence is accumulating that these elicitors act as effectors that specifically interfere with the host defence mechanisms to increase the virulence of pathogens in the absence of the cognate R protein (Abramovitch *et al.*, 2006a; Alfano and Collmer, 2004; He *et al.*, 2007; Mudgett, 2005). To reach this goal, as mentioned above for sumoylation effectors regularly modify the PTM status of host proteins, thereby targeting primary and/or secondary defence responses. Here, we describe the virulence function of some effectors of *P. syringae*.

The RIN4 protein is targeted by two effectors from Pst, AvrRpm1 and AvrB, which indirectly induce RIN4 phosphorylation, thereby enhancing the negative regulation of the primary defence response by RIN4, which leads to increased host susceptibility and pathogen virulence (Kim *et al.*, 2005b; Mackey *et al.*, 2002). The effector HopAI1 dephosphorylates AtMPK6 and AtMPK3 in the MAPK cascade through phosphothreonine lyase activity, which is an alternative cleavage of the phosphate from the threonine residue (Zhang *et al.*, 2007). Similarly, the HopPtoD2 effector functions as a protein tyrosine phosphatase downstream of the host MAPKKs (Espinosa *et al.*, 2003). As MAPK cascades are activated in most stress-related responses, the position of interference is strategic given that the effectors might interfere in many signalling cascades. Even more intriguing is the abuse of the MAPK cascade by *Agrobacterium*, the T-DNA of which hitch-hikes with a phosphorylated AtMPK3 substrate, the transcription factor VIP1, into the nucleus thereby circumventing the defence response (Djamei *et al.*, 2007). Effectors not only modify protein phosphorylation but can also modify protein ubiquitination. The effector AvrPtoB, the N-terminal part of which is recognized by the Fen kinase, has a C-terminal E3 ubiquitin ligase domain. AvrPtoB ubiquitinates the Fen kinase and subsequently targets it for degradation, thereby abolishing the recognition of its own N-terminal region (Abramovitch *et al.*, 2006b; Janjusevic *et al.*, 2006; Rosebrock *et al.*, 2007). AvrPtoB will probably not prove to be the only effector that mediates ubiquitination of host proteins, as a screen of the available bacterial genomes revealed several new putative effectors that are predicted to mimic subunits of the ubiquitination pathway (Angot *et al.*, 2007).

CONCLUDING REMARKS

Rapid PTMs of proteins in defence signalling are essential tools for plants to respond swiftly to pathogen invasion. In this review, we have given an overview of PTMs that modify components essential for defence signalling at the site of signal perception, during secondary messenger production and during signalling in the cytoplasm. PTMs regulate protein localization and activity and provide complex mechanisms to balance responses in the cell without the prerequisite of protein synthesis. As recent technological developments allow high-throughput analysis of modified proteins, we expect that many previously unidentified components of defence signalling cascades, which are not transcriptionally regulated, will be revealed in the coming years.

ACKNOWLEDGEMENTS

We thank Wladimir I. L. Tameling for critically reading the manuscript, and Bart P. H. J. Thomma and Pierre J. G. M. de Wit for helpful comments. I.J.E.S. and M.H.A.J.J. are supported by the Dutch Organization for Scientific Research (NWO; VIDI grant to M.H.A.J.J.).

NOTE

Recently it was shown that AvrPto of *Pseudomonas syringae* inhibits plant immunity triggered by diverse MAMPs. The bacterial effector suppresses early defense gene transcription and intercepts MAPK signaling upstream of MAPKKK at the plasma membrane, linked to the MAMP receptor (He *et al.*, 2006). Xiang and co-workers (Xiang *et al.*, 2008) studied the mechanism behind this observation and found that AvrPto binds the MAMP-triggered receptor-kinases FLS2 and EFR. Targeting of these receptors is required for the virulence function of AvrPto and inhibits FLS2 and EFR kinase activity. As a result of this, MAPK cascade activation is inhibited and MAMP-induced immune responses are suppressed.

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