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Signaling Mechanisms in Pattern-Triggered Immunity (PTI)

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ABSTRACT

In nature, plants constantly have to face pathogen attacks. However, plant disease rarely occurs due to efficient immune systems possessed by the host plants. Pathogens are perceived by two different recognition systems that initiate the so-called pattern-triggered immunity (PTI) and effector-triggered immunity (ETI), both of which are accompanied by a set of induced defenses that usually repel pathogen attacks. Here we discuss the complex network of signaling pathways occurring during PTI, focusing on the involvement of mitogen-activated protein kinases.

Key words: plant defenses, plant immunity, PTI, signaling mechanisms, MAPKs

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INTRODUCTION

Plants are able to convert sunlight into sugar and thus constitute a rich source of carbohydrates and energy for a large variety of organisms, including microbial organisms specialized in attacking plant hosts above and below the ground. Phytopathogens are referred to as biotrophic (e.g. the oomycete *Hyaloperonospora arabidopsidis*), hemibiotrophic (e.g. the bacterium *Pseudomonas syringae*), or necrotrophic (e.g. the fungus *Botrytis cinerea*), depending on their infection and feeding strategy, ranging from feeding on living host cells to killing plant cells to feed on their contents (Kemen and Jones, 2012). Phytopathogens may ultimately reduce the biomass, decrease fertility, or even kill the plant and thus constitute a human concern, as they can decrease the quantity and quality of crop production.

Plants are generally resistant to the majority of pathogens. This phenomenon is termed non-host resistance. Plants are first protected against pathogens by two preformed physical barriers, the cuticle and the cell wall, and by constitutively produced antimicrobial compounds. The cuticle is present on the external surface of the aerial epidermis of all land plants and is mainly composed of cutin and waxes (Yeats and Rose, 2013). It plays important roles in plant physiology, such as to reduce water loss or protect against UV radiation. The cuticle also constitutes a barrier against phytopathogens and pests, although most fungal pathogens can penetrate the cuticle by mechanical rupture and secretion of cutinases that hydrolyze the cutin polyester (Mendgen et al., 1996; Longhi and Cambillau, 1999). The plant cell wall, in addition, is like an exoskeleton surrounding the plant cell and consists of cellulose microfibrils, pectin, hemicelluloses, proteins, and in certain cases, lignin (Somerville et al., 2004). It provides both structural support and protection against biotic and abiotic stresses and is under the control of a dedicated cell wall integrity maintenance mechanism (Hamann, 2012). The combination of cuticle and plant cell wall may be permeable to some fungal pathogens, but bacteria cannot directly penetrate the plant epidermis. Conversely, there are natural openings used by some bacteria to enter the plant: the hydathodes, the nectarthodes, the lenticels, and most importantly, the stomata (Melotto et al., 2008). In addition, plant wounds, due to pests, herbivores, wind, or rainstorms, constitute other routes of plant infection. Beside the physical barriers against pathogen penetration, plants constantly produce antimicrobial compounds (generically called phytoanticipins) that inhibit pathogen growth (Osbourn, 1996). Several proteins have such properties as well as some metabolites, such as glucosinolates and their derivatives, which are secondary metabolites produced in Brassicaceae (Tierens et al., 2001). The few successful pathogens that can breach the preformed barriers then have to face the plant immune system, which is constituted of sophisticated mechanisms of pathogen recognition and defense.

The first tier of the plant immune system corresponds to pathogen perception via the recognition of conserved pathogen-associated molecular patterns (PAMPs) by plant pathogen- or pattern-recognition receptors (PRRs). As PAMPs are

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often also derived from non-pathogens, they are preferentially named microbe-associated molecular patterns (MAMPs) (Ausubel, 2005). The recognition of MAMPs by the plant induces the PAMP-triggered immunity or pathogen-triggered immunity or pattern-triggered immunity (PTI) or MAMP-triggered immunity, a complex set of responses intended for resisting against a pathogen attack. The plant is also able to detect damage-associated molecular patterns (DAMPs), which are plant degradation products resulting from the action of invading pathogens, or endogenous peptides, constitutively present or newly synthesized, which are released by the plants following pathogen attacks (Boller and Felix, 2009). Recognition of DAMPs also triggers immune responses similar to the PTI response (Yamaguchi and Huffaker, 2011). Pathogen perception can also occur via the recognition of pathogen effectors, which are molecules synthesized by the pathogens and delivered in the extracellular matrix or into the plant cell to enhance pathogen fitness by, for example, counteracting the induction of PTI. Plants that are not able to detect these effectors are susceptible to a pathogen, resulting in effector-triggered susceptibility, while plants that can recognize the effectors via disease resistance proteins (R proteins) can implement an immune response called effector-triggered immunity (ETI). The co-evolution of pathogens and plants and notably their repertoire of effectors and R proteins led to the so-called zigzag model (Jones and Dangl, 2006).

After pathogen detection, plants are able to induce a number of defense mechanisms, including stomatal closure to limit entry of bacteria (Melotto et al., 2008; Sawinski et al., 2013), restriction of nutrient transfer from the cytosol to the apoplast to limit bacterial multiplication (Chen et al., 2010; Wang et al., 2012), production and secretion of antimicrobial compounds including phytoalexins, such as camalexin, and defense-related proteins/peptides, such as PR1 (Cowan, 1999; van Loon et al., 2006; Ahuja et al., 2012; Bednarek, 2012), generation of reactive oxygen species (ROS), which have toxic effects on pathogens (O'Brien et al., 2012), and a programmed cell death (PCD), referred to as the hypersensitive response, at the site of infection to limit pathogen progression (Mur et al., 2008).

Upon pathogen recognition, the induction of these defense mechanisms relies on a complex network of signaling pathways. This review discusses the molecular mechanisms that constitute the signaling network occurring during PTI and emphasizes the involvement of mitogen-activated protein kinases (MAPKs). For more clarity and due to space limitations, the review is mainly limited to the *Arabidopsis thaliana*-bacterial pathogen system.

MAMP/DAMP Detection

MAMPs are microbe-derived molecules that are essential for microbes but that can be recognized by plants. MAMPs include proteins (e.g. bacterial flagellin and elongation factor Tu), carbohydrates (e.g. fungal chitin), lipopolysaccharides, etc. (Felix et al., 1999; Kunze et al., 2004; Albert, 2013). DAMPs are plant degradation products generated by the action of pathogen enzymes during the infection process, such as cutin monomers (Yeats and Rose, 2013; Serrano et al., 2014) and cell wall damage products (Hamann, 2012), or endogenous peptides, such as AtPep1, which is derived from its precursor PROPEP1 (Huffaker et al., 2006). PRRs are usually plasma membrane-bound receptor-like kinases (RLKs) or receptor-like proteins with extracellular domains allowing MAMP/DAMP perception (Bohm et al., 2014). A few PRR/MAMP pairs have been identified to date, such as *Arabidopsis* flagellin-sensitive 2 (FLS2), which recognizes the N terminus of flagellin, represented by the 22 amino acid long epitope flg22 from *Pseudomonas aeruginosa* (Gomez-Gomez and Boller, 2000) (Figure 1) and *Arabidopsis* EF-Tu receptor (EFR), which perceives elongation factor Tu (EF-Tu) via an 18 amino acid long eliciting epitope called elf18 from *Escherichia coli* (Zipfel et al., 2006). Similarly for DAMPs, the RLK protein PEPR1 was identified as the receptor of the DAMP AtPep1 (Yamaguchi et al., 2006).

In addition, some proteins form immune receptor complexes with PRRs and are necessary for a normal MAMP perception and transduction of the signal (Monaghan and Zipfel, 2012). MAMP perception induces rapid (in a few seconds) immune receptor complex formation at the plasma membrane and different auto- and trans-phosphorylation reactions of the actors (Macho and Zipfel, 2014). BRI1 associated receptor kinase 1 (BAK1) was shown to interact with FLS2 and its absence caused a reduction of early flg22-dependent responses (Heese et al., 2007). BAK1 can actually interact with different PRRs and bind flg22 in association with FLS2, and can thus be indexed as a co-receptor (Roux et al., 2011; Segonzac and Zipfel, 2011; Sun et al., 2013). In addition to BAK1, the receptor-like cytoplasmic kinase *Botrytis*-induced kinase 1 (BIK1) and related PBL (PBS1-like) kinases constitutively associate with FLS2 and EFR and become quickly phosphorylated and released from the PRR complexes upon MAMP binding (Lu et al., 2010; Zhang et al., 2010).

Ca^{2+} Burst

One of the earliest known physiological response to MAMP/DAMP perception is an influx of extracellular Ca^{2+} in the cytosol (Ca^{2+} burst), which occurs at ~30 s to 2 min and reaches a peak around 4–6 min (Jeworutzki et al., 2010; Ranf et al., 2011; Nomura et al., 2012). It is positively regulated by BIK1 and presumably additional BIK1 family proteins (Li et al., 2014b). This Ca^{2+} influx induces the opening of other membrane transporters (influx of H^+, efflux of K^+, Cl^-, and NO_3^-), which lead to an extracellular alkalinization (~1 min) and a depolarization of the plasma membrane (1–3 min) (Jeworutzki et al., 2010). Generally, cytosolic Ca^{2+} concentrations are regulated by plasma membrane and endomembrane Ca^{2+} channels that mediate the influx of Ca^{2+} and efflux transporters that re-establish Ca^{2+} homeostasis, but in the context of MAMP/DAMP perception and signal transduction, the molecular components involved are still poorly described (Spalding and Harper, 2011). Notably, the plasma membrane *Arabidopsis*-autoinhibited Ca^{2+}-ATPase 8 (ACA8) associates with FLS2 and ACA8 and its closest homolog ACA10 participate in the fine regulation of Ca^{2+} levels during MAMP responses (Frei dit Frey et al., 2012). In addition to the rapid Ca^{2+} transient in the cytosol upon MAMP perception, Nomura et al. (2012) observed a following long-lasting increase (at ~t_0 + 8 min to more than t_0 + 30 min in the case of flg22) of free Ca^{2+} level in the chloroplast stroma. These

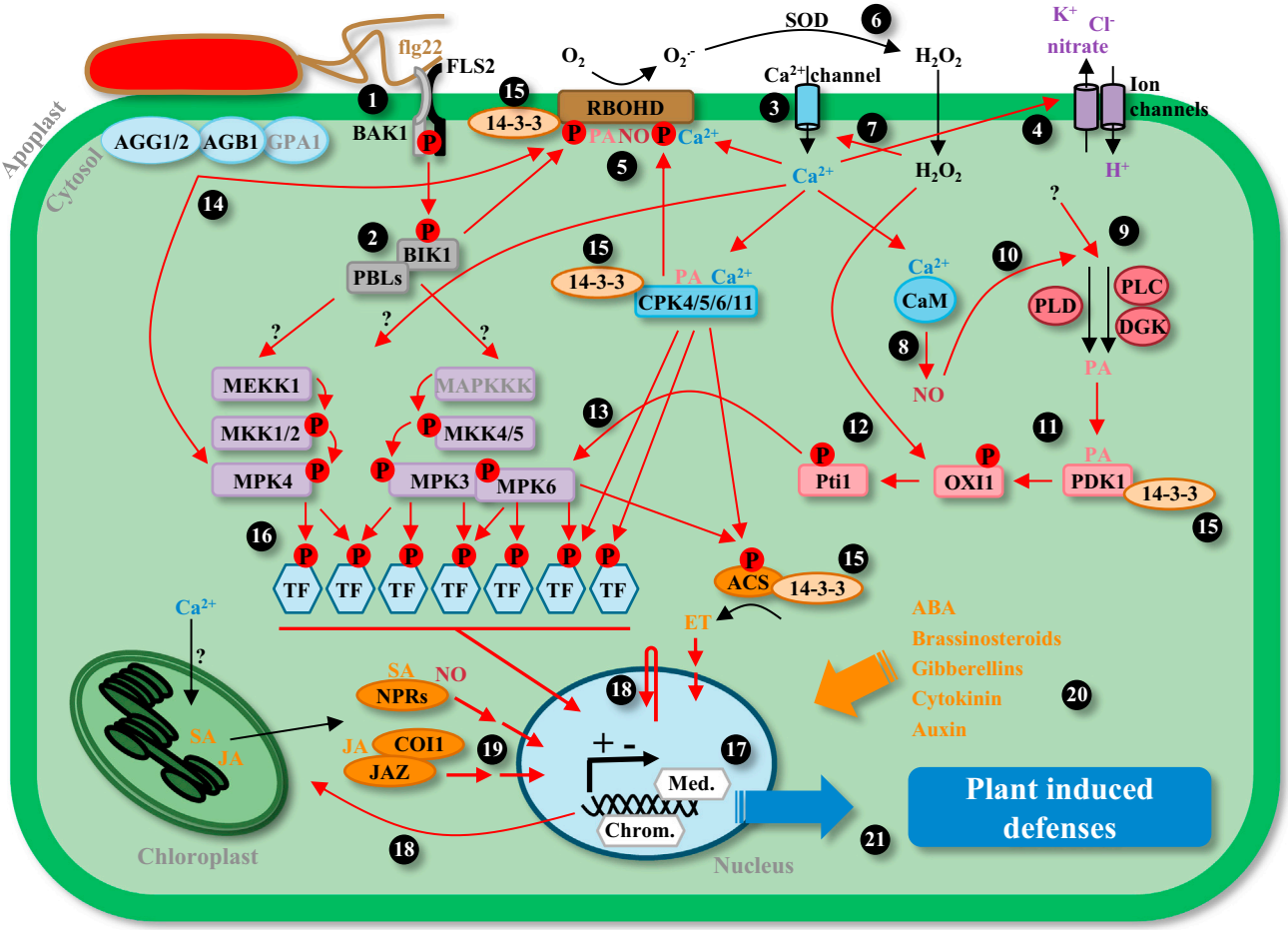

Figure 1. Signaling Mechanisms in PTI.

Flg22 perception by FLS2 PRR induces rapid (seconds) immune receptor complex formation at the plasma membrane and different auto- and trans-phosphorylations of the actors (1). BIK1 becomes quickly phosphorylated and released from the PRR complex (2). A Ca^{2+} burst occurs (~30 s to 2 min) and reaches a peak at around 4–6 min (3). This Ca^{2+} influx induces opening of other membrane channels (influx of H^+, efflux of K^+, Cl^- and nitrate), which lead to an extracellular alkalinization (~1 min) and a depolarization of the plasma membrane (1–3 min) (4). A long-lasting increase (8 to more than 30 min) of free Ca^{2+} levels also occurs in the chloroplast stroma. An ROS burst then rapidly occurs (~2–3 min) via RBOHD and peaks at around 10–14 min (5). Full activation of RBOHD requires phosphorylation by BIK1 and Ca^{2+}-induced CDPKs (5). Ca^{2+}, PA, and NO also regulate RBOHD through direct binding or modification of the protein (5). RBOHD produces $O_2^{\cdot-}$ in the apoplast, which is converted into H_2O_2 by superoxide dismutases (SOD) (6). H_2O_2 can enter the cytosol and the different organelles of the cell and is capable of inducing cytosolic Ca^{2+} elevations (7). Ca^{2+}-induced CaM leads to synthesis of NO, which can regulate NPR1 and RBOHD via cysteine *S*-nitrosylation (8). PA production by PLD and PLC/DGK is rapidly induced (already at 2 min) and reaches a maximum at around 8 min (9). NO production is partly required for PA generation (10). PA can modulate the activity of CDPKs, PDK1, and RBOHD. PA activates OXI1 in a PDK1-dependent manner (11), and H_2O_2 can activate Pti1 kinases in an OXI1-dependent way (12). Pti1 kinases then may regulate MAPKs (13). AGB1, AGG1, and AGG2 G proteins are partly required for ROS burst and probably also MPK4 activation (14). 14-3-3 proteins modulate the activity of RBOHD, CDPKs, PDK1, and several ACS isoforms (15). MAPK modules are activated in a few minutes, probably in part in Ca^{2+}-burst and BIK1/PBLs dependent ways, and, with Ca^{2+}-induced CDPKs, phosphorylate a large spectrum of substrates such as ACS isoforms, leading to ET synthesis, and transcription factors (TFs) (16). TFs, Mediator subunits (Med.), and chromatin remodelers/modifiers (Chrom.) participate in the regulation of several thousand genes (17). First changes include notably genes involved in SA, JA, and ET signaling, synthesis of antimicrobial compounds and transcription regulatory factors (18). SA, JA, and ET signaling pathways then contribute to following gene regulation (19). Crosstalks also occur with other phytohormones (20). This complex signaling network finally leads to the implementation of plant-induced defenses, such as the production and secretion of antimicrobial compounds and the generation of toxic ROS (21). Black arrows denote enzymatic pathways or transport, and red arrows denote regulation (direct or indirect activation/inhibition). Question marks indicate unidentified or unclear events.

investigators also observed a reduction of both the cytoplasmic and stromal Ca^{2+} oscillations using inhibitors of serine/threonine protein kinases and MAPK kinases (MAPKKs), suggesting a possible involvement of an MAPK cascade in the generation of these Ca^{2+} signals. Concerning the above-mentioned rapid apoplast alkalinization upon MAMP/DAMP perception, it is likely that the inhibition of plasma membrane H^+-ATPases also contributes to it in concert with anion efflux and proton influx (Elmore and Coaker, 2011).

ROS Burst

Upon MAMP perception, an extracellular ROS production often referred to as ROS burst rapidly occurs at ~2–3 min and peaks

around 10–14 min (Chinchilla et al., 2007; Nuhse et al., 2007; Ranf et al., 2011). In *Arabidopsis*, the plasma membrane-localized NADPH oxidase named respiratory burst oxidase homolog D (RBOHD) is responsible for this MAMP-induced ROS burst (Nuhse et al., 2007; Zhang et al., 2007; Ranf et al., 2011). Interestingly, the *P. syringae*-elicited ROS burst in *Arabidopsis* leaves is mediated by the sole FLS2 PRR, and ROS production is first detected around 20 min, peaking around 35–40 min with a lower amplitude compared with flg22-induced ROS (Smith and Heese, 2014). These data suggest that flagellin is the predominant MAMP/DAMP detected over the 80 min time course measured post infection and that the quantity and/or accessibility of flagellin is relatively weak (Smith and Heese, 2014). RBOHD can associate with FLS2 and EFR (Kadota et al., 2014; Li et al., 2014b) and, upon MAMP perception, phosphorylation on different residues by both Ca^{2+}-induced calcium-dependent protein kinases (CDPKs) and BIK1 is required for full activation of the NADPH oxidase (Boudsocq et al., 2010; Dubiella et al., 2013; Kadota et al., 2014; Li et al., 2014b). Ca^{2+} itself would also regulate RBOHD through direct binding to the N-terminal EF-hand motifs of the protein (Ogasawara et al., 2008). RBOHD produces membrane-impermeable superoxide $O_2^{\cdot -}$ in the apoplast, which is converted into hydrogen peroxide (H_2O_2) by superoxide dismutases. H_2O_2 is membrane permeable and can enter the cytosol and the different organelles. ROS, like H_2O_2, are also capable of inducing cytosolic Ca^{2+} elevations (Pei et al., 2000; Rentel and Knight, 2004), and MAMP/DAMP-induced ROS burst has a positive feedback effect on cytosolic Ca^{2+} levels by inducing an additive cytosolic Ca^{2+} elevation, leading to a second peak or prolonged plateau (with a maximum at ~5 min after flg22 elicitation) (Ranf et al., 2011). Interestingly, CDPK-dependent phosphorylation of RBOHD also occurs upon ROS stimulation (Dubiella et al., 2013). There exist contradictory results concerning the MPK3/MPK6 dependency of MAMP/DAMP-induced ROS burst. For example, Zhang et al. (2007) showed that inducible expression of the bacterial effector HopAI1, which inactivates MPK3 and MPK6, totally suppressed the flg22-induced ROS burst, suggesting that MPK3/MPK6 act upstream of the ROS burst. However, this approach did not allow distinguishing between the specific contributions of each MAPK. It was then shown that HopAI1 can also inactivate MPK4 (Zhang et al., 2012). It may also be possible that HopAI1 targets another component of flg22-triggered signaling acting upstream of the ROS burst, as it is known that some pathogen effectors can target different signaling elements, such as HopF2, which can target several MKKs and BAK1 (Wang et al., 2010b; Wu et al., 2011; Zhou et al., 2014). Using *mpk* single mutants, Ranf et al. (2011) showed that two different flg22-treated *mpk3* mutants displayed a prolonged ROS burst, while an *mpk6* mutant behaved like wild-type, suggesting that MPK3 negatively regulates the flg22-induced ROS burst. Galletti et al. (2011) showed that the flg22-induced ROS burst is not affected in two independent lines overexpressing AP2C1, which is a MAPK phosphatase inactivating MPK3 and MPK6, suggesting that the ROS burst is independent of MPK3/MPK6. In addition, as mentioned by Galletti et al., MPK4 is also a target of AP2C1 (Schweighofer et al., 2007). Recently, Xu et al. (2014) demonstrated by a genetic approach that an *mpk3 mpk6* double mutant showed similar ROS burst levels as wild-type, suggesting that the ROS burst is independent of MPK3/MPK6. There are also contradictory results concerning the MPK4 dependency of the MAMP/DAMP-induced ROS burst. Zhang et al. (2012) showed that the flg22-induced ROS burst is independent of the MEKK1-MKK1/MKK2-MPK4 cascade. However, independent lines expressing a constitutively active form of MPK4 and *mpk4* mutant plants showed a reduced and an increased ROS burst compared with wild-type, respectively, suggesting that MPK4 negatively regulates ROS burst (Berriri et al., 2012; Xu et al., 2014). It seems clear that the MAMP-induced ROS burst is due to RBOHD and that other RBOH proteins are involved in different signaling pathways (Torres and Dangl, 2005). However, NADPH oxidases are not the only source for ROS in plant cells, and it becomes more and more obvious that a complex temporal and spatial coordination occurs in plants between ROS sources (chloroplasts, peroxisomes, mitochondria, plasma membrane, etc.) and between ROS and other signals (Gross et al., 2013; Baxter et al., 2014).

Other Small Signaling Molecules: Reactive Nitrogen Species and Lipids

Besides Ca^{2+} and ROS, other small molecules are involved in MAMP signaling. Nitric oxide (NO) and its derivatives, collectively referred to as reactive nitrogen species, were shown to be involved at different steps of signal transduction, for example via the regulation of non-expresser of *PR* genes 1 (NPR1) (a master regulator of defense gene expression) oligomeric state by cysteine *S*-nitrosylation, or via the inhibition of RBOHD by cysteine *S*-nitrosylation (Tada et al., 2008; Yun et al., 2011). This and other evidence suggests a close relationship between ROS and NO signaling during pathogen responses (Gross et al., 2013; Scheler et al., 2013). Concerning the lag phase of NO generation, cryptogein and chitosan, a deacylated derivative of chitin, induced NO production in just a couple of minutes, and in the case of chitosan, NO production increased constantly until the last measured time point (60 min) (Foissner et al., 2000; Raho et al., 2011). Pathogen-induced Ca^{2+} influx into the cytosol activates calmodulin (CaM) and/or CaM-like proteins, which then lead to downstream NO synthesis induction (Ma et al., 2008). The origin of NO biosynthesis remains, however, largely unclear (Gupta et al., 2011).

Some lipids, such as phosphatidic acid (PA) and ceramides, were proposed to function as signaling molecules upon pathogen infection (Okazaki and Saito, 2014). Upon flg22 or fungal elicitors (xylanase and *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose), PA production is rapidly induced in tomato cells (already at 2 min for flg22) and reaches a maximum in a few minutes (around 8 min for flg22); in parallel, the levels of the PA precursors PIP_2 and PIP decline after 2 min and the level of DGPP (the phosphorylated product of PA) increases (van der Luit et al., 2000). MAMP-induced NO production is partly required for PA generation via both the phospholipase D (PLD) and phospholipase C/diacylglycerol kinase (PLC/DGK) pathways (Raho et al., 2011). PA can interact with known components of immune signal transduction and modulate their activities, such as CDPKs (Farmer and Choi, 1999; Szczegielniak et al., 2005), 3′-phosphoinositide-dependent protein kinase 1 (PDK1) (Anthony et al., 2004), constitutive triple response 1 (Testerink et al., 2007) and RBOHD/F (Zhang et al., 2009). Exogenous addition of PA induces an ROS burst in tobacco cells in just a few seconds to reach a peak around 6 min (de Jong et al., 2004) and induces the activation of an MAPK in suspension-cultured soybean cells already at 2 min

(Lee et al., 2001). PA can regulate signal transduction at multiple levels, such as ROS production (Zhang et al., 2009; Nakano et al., 2013), MAPK activity (Testerink et al., 2007), jasmonic acid (JA) production (Wang et al., 2000; Nakano et al., 2013) and possibly ethylene (ET) production (Testerink et al., 2007, 2008).

Heterotrimeric G Proteins

Arabidopsis has one canonical Gα subunit (GPA1), one Gβ subunit (AGB1), and three Gγ subunits (AGG1, AGG2, and AGG3) (Urano and Jones, 2014). Contrary to their animal counterparts, plant G proteins are independent of G protein–coupled receptors and seemingly self-activating (Urano and Jones, 2014). Whereas GPA1 is not required for basal disease resistance to *P. syringae* and has no obvious effect on cell death (Liu et al., 2013a; Torres et al., 2013) or MAMP-induced MPK3/MPK6 activation (Ishikawa, 2009), AGB1, AGG1, and AGG2 play important roles. For example, MAMP- and *P. syringae*-induced ROS production is reduced in *agb1* (Liu et al., 2013a; Lorek et al., 2013; Torres et al., 2013). Whereas *agg1* and *agg2* single mutants do not show a phenotype, the *agg1 agg2* double mutant is more susceptible to *P. syringae* and impaired in flg22-, elf18-, and chitin-induced resistance (Liu et al., 2013a). MPK3 and MPK6 activation are not altered in *agb1* or *agg1 agg2* (Ishikawa, 2009; Liu et al., 2013a), but MPK4 activation is slightly reduced (Liu et al., 2013a). Overall, these results suggest that AGB1, AGG1, and AGG2, but not GPA1, are required for MAMP-triggered immunity mediated by the RLKs FLS2, EFR, and CERK1. These G proteins are also partly required for MAMP-induced ROS burst, but MAMP-induced activation of MPK3, 4, and 6 is mainly independent of G proteins. How AGB1-AGG1/2 dimers are activated by RLKs is still undetermined. The precise way AGB1-AGG1/2 dimers regulate downstream responses is also unclear.

14-3-3 Proteins

14-3-3 proteins also participate in immune signal transduction. There are 15 members of the 14-3-3 gene family in *Arabidopsis*, and 14-3-3 proteins usually interact with phosphorylated proteins, their clients, to help them complete their activation (Sehnke et al., 2002; Chevalier et al., 2009). A number of reports demonstrated the involvement of 14-3-3 proteins in disease resistance in different plant species, generally underlying a positive role for these proteins in pathogen defense (Yang et al., 2009; Oh et al., 2010; Manosalva et al., 2011; Oh and Martin, 2011; Taylor et al., 2012; Lozano-Duran et al., 2014; Teper et al., 2014). 14-3-3 proteins were shown to interact with known components of immune signal transduction and modulate their activity, such as maize plasma membrane H^+-ATPase (Jahn et al., 1997), NtRBOHD (Elmayan et al., 2007), tomato MAPKKKalpha and MKK2 (Oh et al., 2010; Oh and Martin, 2011), or the *Arabidopsis* factors CPK-1 (CDPK) (Camoni et al., 1998), PA, and PDK1 (Testerink et al., 2004; Otterhag et al., 2006) and several 1-aminocyclopropane-1-carboxylic acid synthase (ACS) isoforms involved in ET biosynthesis (Yoon and Kieber, 2013). These different studies suggest the involvement of 14-3-3 proteins both in PTI and ETI. A recent report illustrates the role of 14-3-3 proteins in PTI. Chemical disruption of 14-3-3 protein interactions with their client proteins, using 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), drastically reduced the MAMP-triggered ROS burst and stomatal closure in *Arabidopsis* and *Nicotiana benthamiana* (Lozano-Duran et al., 2014). The 14-3-3 protein GRF8/AtMIN10 is destabilized by the *P. syringae* effector HopM1 and preferentially the membrane-associated pool of GRF8 protein (Nomura et al., 2006). Lozano-Duran et al. (2014) showed that the virulence of a *P. syringae* strain lacking the effector HopM1 was restored using AICAR, further confirming a role for 14-3-3 proteins during immune responses. These authors also showed a reduction of MAMP-triggered ROS burst in the knockdown mutants of tomato and *N. benthamiana* orthologs of GRF8/AtMIN10. The current data indicate that 14-3-3 proteins can interact with many phosphorylated proteins involved at different steps of the plant immune signal transduction, but the full extent of their implication and their mechanisms of action require further investigation.

Protein Kinases

Protein phosphorylation, mediated by protein kinases and removed by protein phosphatases, is probably the most abundant post-translational modification found in eukaryotes and plays important functions in signal transduction (Minguez et al., 2012; Olsen and Mann, 2013). Phosphorylation of proteins can affect important properties, such as protein stability, enzyme activity, or subcellular localization (Bigeard et al., 2014). Numerous phosphorylation events occur upon MAMP perception, and phosphoproteomic approaches revealed their extent and allowed some of the protein phosphorylation sites to be identified precisely (Benschop et al., 2007; Nuhse et al., 2007; Mithoe et al., 2012; Rayapuram et al., 2014). The decreased abundance of some phosphosites upon MAMP treatment also suggests that phosphatases may play a preeminent role in PTI signaling (Nuhse et al., 2007; Rayapuram et al., 2014).

Besides the previously mentioned auto- and trans-phosphorylation of immune receptor complexes occurring in seconds, other protein kinases rapidly become activated upon MAMP perception. The majority of them belong to the CDPK, MAPK, and AGC (Protein kinase A, G and C) protein kinase families, and they are thought to be key elements allowing regulation of a large spectrum of protein targets, such as transcription factors, metabolic enzymes, plasma membrane proteins, and cytoskeleton proteins (Nuhse et al., 2007; Rayapuram et al., 2014).

CDPKs

The genome of *Arabidopsis* encodes for 34 CDPKs (Cheng et al., 2002). CDPKs have an N-terminal serine/threonine protein kinase domain and a C-terminal CaM-like domain with EF-hand calcium-binding sites and most of them are clearly Ca^{2+}-dependent (Cheng et al., 2002; Boudsocq and Sheen, 2013). Some CDPKs were shown to be transiently activated between 5 and 30 min upon flg22, such as CPK4, CPK5, CPK6, and CPK11 (Boudsocq et al., 2010). CPK4/5/6/11 are involved in flg22-mediated ROS burst, flg22-mediated transcriptional reprogramming, and flg22-induced resistance to the bacterial pathogen *P. syringae* (Boudsocq et al., 2010; Romeis and Herde, 2014). Several CDPK substrates were identified, such as RBOHD as a substrate of CPK5, and tomato ACS2 as a substrate of tomato CDPK2 (Kamiyoshihara et al., 2010; Boudsocq and Sheen, 2013; Dubiella et al., 2013; Schulz et al., 2013). Besides Ca^{2+}, some CDPKs can also be regulated by other molecules themselves involved in signal transduction, such as lipids and

14-3-3 proteins (Camoni et al., 1998; Farmer and Choi, 1999; Szczegielniak et al., 2005; Klimecka and Muszynska, 2007; Lachaud et al., 2013).

AGC and Related Kinases

AGC protein kinases have been shown to participate in plant immune responses and regulate pathogen-induced MAPK cascades (Garcia et al., 2012). In *Arabidopsis*, the AGC kinase family contains 39 members (Rademacher and Offringa, 2012). AGC2-1, also named OXI1, is required for complete activation of MPK3 and MPK6 by H_2O_2 and cellulase treatments and for resistance against the pathogens *Hyaloperonospora arabidopsidis* and *P. syringae* (Rentel et al., 2004; Petersen et al., 2009). OXI1 gene expression and kinase activity are rapidly induced by MAMPs and DAMPs, such as treatment with flg22, cellulase, and H_2O_2 in leaves and roots (Rentel et al., 2004; Jacobs et al., 2011). Interestingly, OXI1 transcriptional induction is reduced in mutants of RBOHD or by treatment with the NADPH oxidase inhibitor, diphenylene iodinium (Petersen et al., 2009). RBOHD is responsible for the ROS burst upon treatment with MAMPs and biotrophic pathogens (Nuhse et al., 2007; Zhang et al., 2007; Ranf et al., 2011), indicating that the production of ROS by RBOHD mediates OXI1 induction during plant–pathogen interactions. OXI1 regulates a group of protein kinases named Pti1-1 to Pti1-4 due to their similarity with tomato Pti1 (Anthony et al., 2006; Forzani et al., 2011). The stress-inducible Pti1–4 was found to interact with MPK6 *in vivo* (Forzani et al., 2011). As other AGC kinases, OXI1 activity is regulated by the AGC kinase PDK1, and PA was reported to activate OXI1 in a PDK1-dependent manner (Anthony et al., 2004). However, H_2O_2 and flg22 lead to PDK1-independent but OXI1-dependent activation of Pti1 kinases (Anthony et al., 2006), suggesting that other protein kinases function upstream of OXI1 upon stimulation by ROS or MAMPs.

MAPKs

The *Arabidopsis* genome codes for 20 MAPKs, 10 MAPKKs, and 60 MAPKKKs (MAPK-Group, 2002). MAPKKKs, MAPKKs, and MAPKs constitute functional signaling modules, which translate extracellular stimuli into appropriate responses. They were largely studied in the context of plant–pathogen interactions (Meng and Zhang, 2013). Upon flg22 and other MAMP treatments, four MAPKs (MPK3, MPK4, MPK6, and MPK11) are activated within 1–2 min to reach a peak of activity at 10–15 min, which then rapidly decreases (Nuhse et al., 2000; Zipfel et al., 2006; Bethke et al., 2012). Two signaling modules are composed of MKK4/MKK5 activating MPK3/MPK6 by phosphorylation (Asai et al., 2002; Ren et al., 2002) and MEKK1-MKK1/MKK2 upstream of MPK4 (Figure 2) (Ichimura et al., 1998; Mizoguchi et al., 1998; Huang et al., 2000; Petersen et al., 2000; Matsuoka et al., 2002; Teige et al., 2004; Hadiarto et al., 2006; Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007; Gao et al., 2008; Qiu et al., 2008b; Berriri et al., 2012). MEKK1 was initially proposed to be the MAPKKK upstream of MKK4/MKK5-MPK3/MPK6 (Asai et al., 2002). However, upon flg22 treatment, MPK3 and MPK6 were shown to still be activated in *mekk1* mutant, suggesting that MEKK1 is not the upstream MAPKKK of the module or that at least one other MAPKKK plays a redundant role (Ichimura et al., 2006; Suarez-Rodriguez et al., 2007). Interestingly, a MAPK module involved in stomatal development and patterning and in inflorescence architecture consists of MKK4/MKK5-MPK3/MPK6 downstream of YODA, a MAPKKK (Wang et al., 2007; Meng et al., 2012). Yet, MAMP-triggered activation of MPK3 and MPK6 seems to be normal in a *yoda* mutant, according to unpublished results from Liu and Zhang, cited in Meng and Zhang (2013). The identity of the MAPKKK(s) upstream of MKK4/MKK5-MPK3/MPK6 in the context of immunity is thus still unclear. The MKK4/MKK5-MPK3/MPK6 module positively regulates defense responses (Pitzschke et al., 2009; Rasmussen et al., 2012; Zhao et al., 2014). Concerning the second module, a kinase-impaired version of MEKK1 was able to rescue the *mekk1* dwarf phenotype, suggesting that MEKK1 would have a structural role independent of its enzymatic activity, but it is nonetheless not totally excluded that this MEKK1 version has residual activity (Suarez-Rodriguez et al., 2007). In yeast two-hybrid analysis, MEKK1 can also directly interact with MPK4, which led to the proposition that MEKK1 may serve as a scaffold protein (Ichimura et al., 1998). However, no further data have supported this hypothesis yet. At first, the MEKK1-MKK1/MKK2-MPK4 module was thought to negatively regulate defense responses, because the mutants of this cascade present constitutive defense responses, such as accumulation of H_2O_2 and callose and constitutive expression of *PR* genes, and are more resistant to pathogens. However, recent reports showed that this MAPK module actually positively regulates defense but is guarded by the R protein suppressor of *mkk1 mkk2*, 2 (SUMM2) via the regulation of suppressor of *mkk1 mkk2*, 1 (SUMM1) which is the MAPKKK MEKK2 (Kong et al., 2012a; Zhang et al., 2012; Su et al., 2013).

The molecular connection between the PRR immune complexes and the downstream MAPKKKs remains to be elucidated. Some data suggest that flg22-induced MPK3, MPK4, and MPK6 activation is in part dependent on the Ca^{2+} burst (Boudsocq et al., 2010; Ranf et al., 2011) but, flg22-induced MPK3, MPK4, and MPK6 activation is not changed in a *cpk5 cpk6 cpk11* triple mutant, and constitutively active forms of CPK4, CPK5, CPK6, and CPK11 do not activate MPK3 and MPK6 in protoplasts, suggesting that MAPK activation is independent of CDPKs (Boudsocq et al., 2010). Flg22-induced MPK3, MPK4, and MPK6 activation is wild-type-like in an *rbohd* mutant, suggesting that an ROS burst is not required for MAPK activation (Zhang et al., 2007; Xu et al., 2014). In addition, flg22-induced activation of MPK3, MPK4, and MPK6 is normal in a *bik1 pbl1* double mutant, suggesting that their activation is not dependent on BIK1 and PBL1 (Feng et al., 2012), but flg22-induced MAPK activation is reduced upon expression of the bacterial AvrAC effector, which prevents the activation of BIK1 and presumably related kinases, suggesting that additional BIK1-related proteins may lead to MAPK activation (Feng et al., 2012). Moreover, using a quadruple mutant (*dde2 ein2 pad4 sid2*) for essential genes of salicylic acid (SA) (*PAD4*, phytoalexin deficient 4; *SID2*, SA induction-deficient 2), JA (*DDE2*, delayed-dehiscence 2) and ET (*EIN2*, ethylene insensitive 2) signaling pathways, Tsuda et al. (2009) concluded that flg22-induced MPK3/MPK6 activation is comparable between wild-type and *dde2 ein2 pad4 sid2* mutants, suggesting that MAPK activation is independent of the SA, JA, and ET signaling pathways.

MAPK Substrates

Currently, an important challenge in biology is to associate with high confidence a given protein kinase to its *bona fide* substrates. This is particularly true in the context of plant signaling. In PTI,

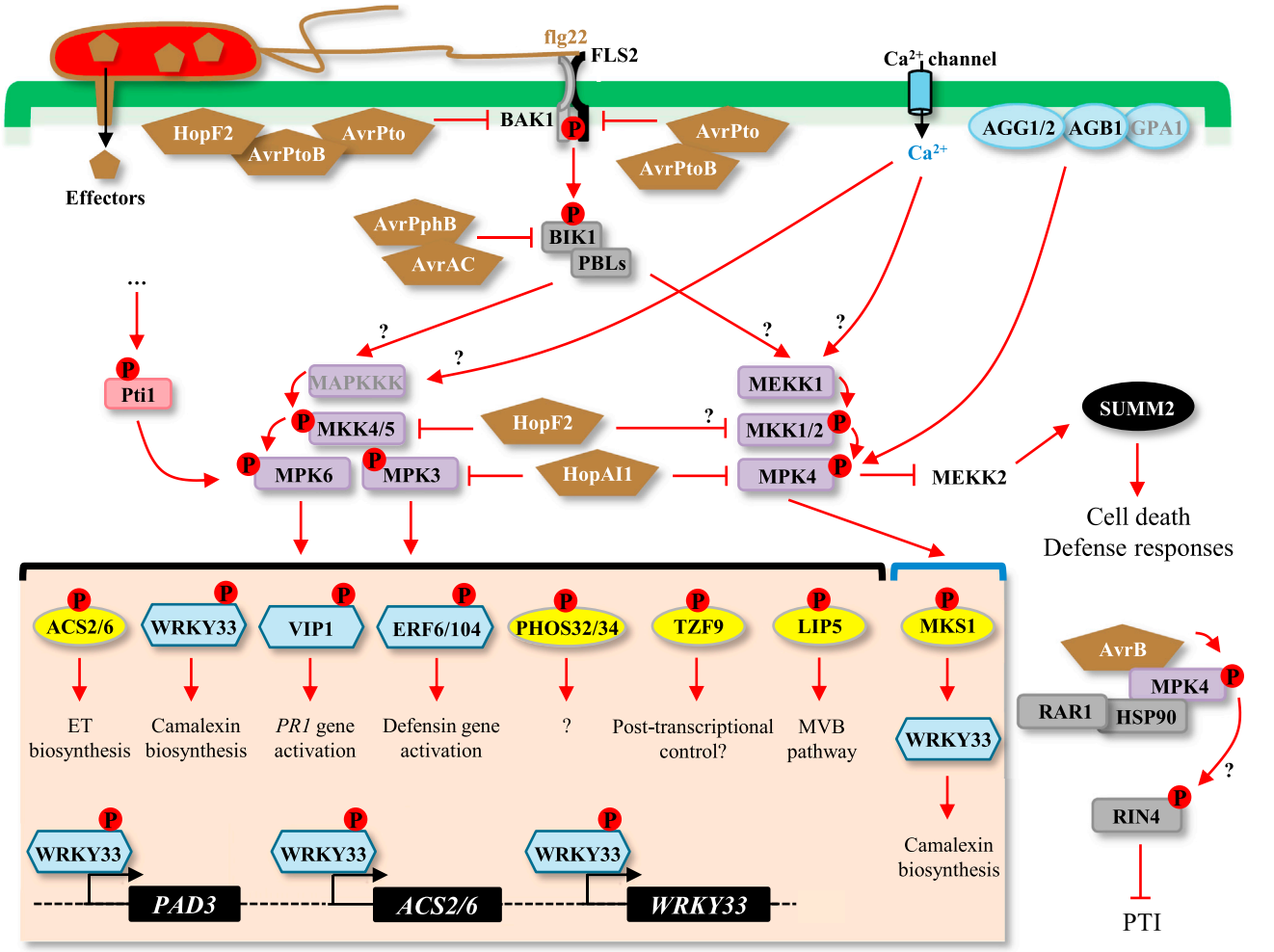

Figure 2. MAPK Modules in *Arabidopsis* Immunity.

Two MAPK modules are involved in plant defense signaling. They are composed of MKK4/MKK5-MPK3/MPK6 and of MEKK1-MKK1/MKK2-MPK4. These two modules are downstream of BAK1/FLS2, but their connection to the PRR immune complex is currently unclear. Nonetheless, some data suggest that MPK3, MPK4, and MPK6 activation is in part dependent on the Ca^{2+} burst and on BIK1/PBLs. The MEKK1-MKK1/MKK2-MPK4 module is also connected to heterotrimeric G proteins, and Pti1 kinases may regulate MAPKs. Several substrates of MPK3, MPK4, and MPK6 have been identified in the context of pathogen response, such as WRKY33 and VIP1 transcription factors. Through phosphorylation, MAPKs induce notably the activation of defense genes and ET biosynthesis, contributing to plant immunity. Several MAPK substrates were also identified in other contexts that could possibly also be involved in pathogen response. Most of these substrates are transcription factors induced upon ET or abiotic stresses and are mentioned in the text. The MEKK1-MKK1/MKK2-MPK4 module is guarded by the R protein SUMM2 through a mechanism of control of MEKK2. Pathogen effectors, such as HopAI1, lead to MPK4 deactivation; MEKK2 control is thus reduced, which activates SUMM2, leading to cell death and other defense responses. MPK4 is also targeted by the effector AvrB, which leads to MPK4 activation and the subsequent activation of RIN4, which is a negative regulator of defense. Effectors actually target many components of defense signaling, such as FLS2, BAK1, BIK1, and related PBLs, and immune MAPK modules. Black arrows denote transport and red arrows denote regulation (direct or indirect activation/inhibition). Question marks indicate unidentified or unclear events.

MAPK substrates have been the most extensively studied, and several substrates have benefited from both biochemical and genetic evidence and can thus be qualified as *bona fide* MAPK substrates.

Transcription Factors

Almost half of *bona fide* immune MAPK substrates are transcription factors, highlighting the involvement of immune MAPKs in the transcriptional reprogramming occurring during defense. MPK3, MPK4, and MPK6 phosphorylate the transcription factor WRKY33 *in vitro*, and the phosphorylation by MPK3 and MPK6 was also demonstrated *in vivo* (Andreasson et al., 2005; Mao et al., 2011). Genetic analyses showed that WRKY33 is essential for camalexin biosynthesis upon different pathogen infections and upon MPK3/MPK6 signaling (Qiu et al., 2008a; Mao et al., 2011). The five potential MAPK phosphorylation sites in WRKY33 were mutated to alanine to obtain the $WRKY33^{SA}$ mutant (Mao et al., 2011). $WRKY33^{SA}$ could not complement the *wrky33* mutant phenotype, and, notably, the activation of camalexin biosynthetic genes was less efficient. In addition, WRKY33 was shown to bind the promoter of *PAD3*, suggesting that it can directly activate the expression of camalexin biosynthetic genes (Qiu et al., 2008a; Mao et al., 2011). Interestingly, MPK3/MPK6 signaling controls the expression of *WRKY33*, and WRKY33 can bind its own

promoter, suggesting that a positive feedback regulatory loop occurs in the regulation of *WRKY33* expression (Mao et al., 2011). Overall, camalexin biosynthesis is thus markedly induced by MPK3/MPK6 signaling via both transcriptional and post-transcriptional regulation of WRKY33. Actually, WRKY33 was also shown to control camalexin biosynthesis through its interaction with MPK4 (Qiu et al., 2008a). WRKY33 forms a nuclear complex with MPK4 and MKS1 (Qiu et al., 2008a). Upon flg22 or bacterial infection, phosphorylation of MKS1 by MPK4 induces the release of the MKS1-WRKY33 complex from MPK4, which then activates *PAD3* gene expression (Andreasson et al., 2005; Caspersen et al., 2007; Qiu et al., 2008a). In addition, MKS1 recognizes the DNA binding WRKY domain of WRKY33 and stimulates its binding to DNA (Lai et al., 2011). However, camalexin accumulation still occurs in *mks1* upon pathogen infection, and *PAD3* expression is constitutive in *mpk4* and is still pathogen induced in *mks1* (Qiu et al., 2008a). In addition, silencing MKS1 partially suppresses *mpk4* dwarfism (Andreasson et al., 2005). Altogether, it thus seems that both MPK3/MPK6 and MPK4-MKS1 signaling regulate WRKY33 for camalexin biosynthesis, but the exact crosstalk mechanism still needs to be clarified.

MPK3 interacts with the bZIP transcription factor VIP1 *in vivo* and phosphorylates it *in vitro* on S79 (Djamei et al., 2007). Use of lines expressing VIP1, VIP1^{S79A}, or VIP1^{S79D} indicated that VIP1 and VIP1^{S79A} localized both in the cytoplasm and in the nucleus, while VIP1^{S79D} localized predominantly in the nucleus. Upon flg22 treatment, VIP1 relocalized to the nucleus, whereas VIP1^{S79A} did not. Further experiments indicated that nuclear VIP1 activated *PR1* gene expression. These results indicate that upon flg22 treatment, MPK3 phosphorylates VIP1, which induces the relocalization of the protein to the nucleus, where it activates *PR1* gene expression (Djamei et al., 2007).

MPK6 interacts with ERF104, a member of the ET response factor family, and the interaction is lost within 5–15 min of flg22 treatment (Bethke et al., 2009). Interestingly, the protein complex disruption requires both MPK6 activity and ET signaling. MPK6 phosphorylated the C-terminal domain of ERF104 *in vitro*. Moreover, transcriptomic analysis of an ERF104 overexpressing line showed increased expression of some stress-related genes, notably *PDF1.2* defensin genes. Overall, the data suggest that upon flg22 treatment, MPK6 phosphorylates ERF104, which induces the release of ERF104 and its activation of defense genes (Bethke et al., 2009). ERF104 seems to be a specific substrate of MPK6, but another ERF transcription factor, ERF6, is phosphorylated by both MPK3 and MPK6 (Meng et al., 2013). *In vitro*, the phosphorylation occurs predominantly on S266 and/or S269, and *in vivo* analyses indicated that ERF6 is phosphorylated on both S266 and S269 in response to *B. cinerea* infection (Meng et al., 2013). In addition, induction of a constitutively active tobacco NtMEK2 also led to phosphorylation of *Arabidopsis* ERF6 S266 and S269 residues, suggesting that MPK3 and MPK6 phosphorylate these sites *in vivo*. Phosphorylation of ERF6 attenuates the proteasome-mediated degradation of ERF6. Moreover, ERF6 expression is strongly induced upon *B. cinerea* infection, and this induction is partly dependent on the MPK3/MPK6 signaling but is ET independent. An ERF6 phospho-mimicking mutant plant showed higher resistance to *B. cinerea* compared with wild-type plants and exhibited a severe dwarfism, which is not similar to the *mpk4* mutant dwarfism, as revealed from the absence of *PR1* transcript and ROS accumulation. On the contrary, a dominant-negative ERF6 mutant plant showed higher sensitivity to *B. cinerea* and was bigger than wild-type plants. The authors also demonstrated that ERF6 functions downstream of MPK3/MPK6 signaling in inducing the expression of several defensin genes, including *PDF1.1* and *PDF1.2a*. Overall, the data indicate that ERF6 is a substrate of MPK3 and MPK6 that participates in defense gene activation and fungal disease resistance (Meng et al., 2013).

Other Substrates

Besides transcription factors, several other immune MAPK substrates were identified, which are involved in diverse cellular functions. MPK6 phosphorylates *in vitro* ACS2 and ACS6, two isoforms of 1-aminocyclopropane-1-carboxylic acid synthase, which is the rate-limiting enzyme of ET biosynthesis (Liu and Zhang, 2004). The phosphorylated sites were identified in ACS6 as S480, S483, and S488. A mutant form of ACS6, ACS6$^{S480D/S483D/S488D}$, became more stable, leading to elevated cellular ACS activity and ET biosynthesis. In addition, genetic analyses showed that both ACS2 and ACS6 were required to produce high levels of ET upon MPK6 activation (Liu and Zhang, 2004). The same group later showed the involvement of MPK3 in the same process (Han et al., 2010) and discovered the precise phosphorylation-dependent mechanism explaining the stability of the ACS proteins or their degradation through the ubiquitin-proteasome machinery (Joo et al., 2008). The data demonstrate that MPK3 and MPK6 regulate ET biosynthesis through the phosphorylation of ACS2 and ACS6. Interestingly, WRKY33 also directly binds to the promoters of *ACS2* and *ACS6 in vivo*, and MPK3/MPK6 signaling induces *ACS2* and *ACS6* gene expression in a way largely dependent on WRKY33 (Li et al., 2012). Thus, similar to camalexin biosynthesis via WRKY33 regulation, MPK3/MPK6 signaling regulates ET production via both the transcriptional and post-transcriptional regulation of ACS2 and ACS6.

The tandem zinc finger protein 9, TZF9, was initially identified as an *in vitro* substrate of MPK3 and MPK6 using protein microarray screening (Feilner et al., 2005). Maldonado-Bonilla et al. (2013) confirmed this result in a classical *in vitro* kinase assay and further showed that both MPK3 and MPK6 can interact *in vivo* with TZF9, in both the cytosol and the nucleus. Experiments in protoplasts transiently expressing TZF9 suggested that TZF9 is phosphorylated upon flg22 treatment and that this phosphorylation may alter TZF9 protein stability. Phenotyping of a *tzf9* mutant showed a partial attenuation of MAMP-induced responses: reduction of ROS burst, of MAPK activation, and of *FRK1* and *NHL10* defense gene expression. Moreover, a *tzf9* mutant exhibited higher susceptibility to *P. syringae*. TZF9 localizes in processing bodies (RNA–protein cytoplasmic structures containing proteins involved in RNA decay and messenger RNAs [mRNAs] targeted to degradation or translationally repressed) and binds RNA *in vitro*, probably via its CCCH-type motifs. The data thus suggest that MPK3 and MPK6 phosphorylate TZF9 during plant defense, which would destabilize the protein and thus alter its probable involvement in post-transcriptional control, which is important to regulate plant immunity (Maldonado-Bonilla et al., 2013).

MPK6 and probably MPK3 interact with LIP5 *in vitro* and *in vivo*, and both MAPKs phosphorylate LIP5 *in vitro* (Wang et al., 2014). LIP5 is also phosphorylated *in vivo* upon pathogen infection and upon MPK3/MPK6 signaling, which stabilizes the protein. Flg22-induced defenses are normal in *lip5* mutant, but basal resistance to *P. syringae* is compromised. Mutation of MAPK potential phosphorylation sites in LIP5 decreases LIP5 stability and compromises the capacity of LIP5 to complement *lip5* mutant. Plants infected by *P. syringae* largely require LIP5 to increase the formation of multivesicular bodies (MVB) and exosome-like paramural vesicles localized between the plasma membrane and the cell wall. Overall, the results suggest that MPK3 and MPK6 phosphorylate LIP5, which stabilizes the protein and positively regulates the MVB pathway, leading probably to the relocalization of defense-related molecules (Wang et al., 2014).

PHOS32 and the related protein PHOS34 were identified as proteins being rapidly phosphorylated upon flg22 treatment, and the phosphorylated site in PHOS32 was identified as S21 (Merkouropoulos et al., 2008). *In vitro* kinase assays showed that MPK3 and MPK6 can phosphorylate PHOS32, predominantly on S21, and immunodepletion assays indicated that MPK3 and MPK6 are the predominant protein kinases phosphorylating this residue. PHOS32 and PHOS34 contain a universal stress protein A domain, named after a bacterial protein that accumulates in response to many stresses. However, neither the exact functions of PHOS32 and PHOS34 in plant defense nor the role of the phosphorylation by MPK3 and MPK6 have been reported yet (Merkouropoulos et al., 2008).

Besides MPK3, MPK4, and MPK6, a function was recently found for MPK11. MPK11 interacts *in vivo* with the 14-3-3 λ isoform GRF6, predominantly in the nucleus (Carrasco et al., 2014). Co-expression experiments in tobacco and use of anti-phosphoSer or anti-phosphoThr antibodies suggest that MPK11 phosphorylates GRF6 on Ser residue(s). Moreover, the decreased abundance of GRF6 upon co-expression with MPK11 suggests an MPK11-mediated destabilization of GRF6. In addition, use of an anti-ubiquitin antibody suggests that GRF6 is degraded via the ubiquitin-proteasome system. Upon potyvirus plum pox virus (PPV) infection, *mpk11* and *grf6* show increased susceptibility and enhanced resistance, respectively. Overall, these data suggest that MPK11 phosphorylates GRF6 leading to its ubiquitin-proteasome degradation and resistance to PPV (Carrasco et al., 2014).

MAPK Substrates in Other Contexts

Several substrates of MPK3, MPK4, and MPK6 were identified in other contexts, either connected to pathogen response (e.g. ET and H_2O_2 treatments), abiotic stress (e.g. salt and heat stress), or other contexts (e.g. development). More than the half of them are transcription factors and could also potentially be *bona fide* substrates of MPK3, MPK4, and MPK6 in the context of plant immunity. These proteins are the transcription factor EIN3 (Guo and Ecker, 2003; Yoo et al., 2008), the nitrate reductase NIA2 (Wang et al., 2010a), the bHLH transcription factor SPCH (Wang et al., 2007; Lampard et al., 2008), the mRNA decapping enzyme DCP1 (Xu and Chua, 2012), the transcription factors MYB41 and MYB44 (Hoang et al., 2012; Nguyen et al., 2012; Persak and Pitzschke, 2013), the transcription factors HSFA2 and HSFA4A (Evrard et al., 2013; Perez-Salamo et al., 2014), the lipid transfer protein-related hybrid proline-rich protein, AZI1 (Pitzschke et al., 2014), the bHLH transcription factor MYC2 (Sethi et al., 2014), the MAPK phosphatase MKP1 (Park et al., 2011; Gonzalez Besteiro and Ulm, 2013), the cytoskeleton protein MAP65-1 (Smertenko et al., 2006; Beck et al., 2010; Kosetsu et al., 2010; Sasabe et al., 2011), and the C2H2-type transcription factors ZAT6 and ZAT10 (Nguyen et al., 2011; Liu et al., 2013b).

Putative MAPK Substrates from Medium-/High-Throughput Analyses

The MAPK substrates described above were mainly identified from candidate approaches. In contrast, many putative substrates were identified as part of medium-/high-throughput analyses, but the majority of them still need to be supported by further evidence. Feilner et al. (2005) produced protein microarrays including 1690 *Arabidopsis* proteins and incubated them with MPK3 and MPK6. Forty-eight putative substrates of MPK3 and 39 of MPK6 were identified, with an overlap of 26 common substrates. Among them was ACS6, a known substrate of MPK3 and MPK6. The other putative substrates were transcription factors (e.g. MYB88), ribosomal proteins (e.g. RPL23A), histones (e.g. H3), and other proteins (e.g. casein-kinase, thioredoxins). Popescu et al. (2009) used protein microarrays containing 2158 *Arabidopsis* proteins, and gene ontology analyses indicated that 50.4% were putative transcription factors, 34% putative nucleic acid-binding proteins, and 16% protein kinases. Ten different *Arabidopsis* MAPKs were tested, including MPK3, MPK4, and MPK6. In total, 570 proteins were identified as MAPK putative substrates. About 140, 150, and 185 proteins were identified as potential substrates of MPK3, MPK4, and MPK6, respectively. Hoehenwarter et al. (2013) reported a phosphoproteomic study on *Arabidopsis* plants expressing the constitutively active tobacco $MEK2^{DD}$ under the control of a DEX-inducible promoter. $MEK2^{DD}$ activates *Arabidopsis* MPK3 and MPK6 *in vivo*. A total of 36 proteins phosphorylated on S/T-P sites were identified as putative MPK3/MPK6 substrates. Among these were known substrates of MAPKs, such as PHOS34 and MKP1, but the majority of the candidates had not been reported previously, including PEARLI4 (a phospholipase-like protein), the plant-specific leucine-rich repeat protein PIRL9, TFIIB, NOT2/3/5, VQ4 (a VQ-motif-containing protein [VQP]), and TIC. Pecher et al. (2014) systematically analyzed the link between *Arabidopsis* MAPKs and VQPs. The VQ-motif, F(R/K)xΦVQxΦTG, is plant specific, and *Arabidopsis* contains 34 genes coding for VQPs (Cheng et al., 2012) including MKS1, which is a substrate of MPK4. A yeast two-hybrid screen indicated that MPK3, MPK4, MPK6, and MPK11 are the major VQP-interacting MAPKs. In addition, 10 VQPs were phosphorylated *in vitro* by MPK3 and MPK6 and were thus renamed MPK3/MPK6-targeted VQPs (MVQs). Some VQPs are known to interact with WRKY transcription factors, such as MKS1 with WRKY33. The interactions between MVQs and WRKYs were thus screened in yeast two-hybrid, and MVQ1 to MVQ10 were found to interact predominantly with group I and group IIC WRKYs. Pecher et al. (2014) showed that MAMP treatment triggered a change in phosphorylation states and/or stability of a subset of MVQs *in vivo*. These authors also demonstrated that the MAMP-triggered phosphorylation of MVQ1 is essentially performed by MPK6 *in vivo*, probably on all 12 potential phosphosites in MVQ1, and that the MAMP-induced *NHL10* gene expression is controlled positively by some WRKYs but negatively by overexpressing MVQ1. Overall,

these results suggest that a variety of WRKY-VQP protein complexes exist and that pathogen-induced transcriptional changes could be modulated, notably by changing the composition of these complexes via MAPK-induced VQP degradation.

MAPK Modules Targeted by Pathogen Effectors

The vast majority of pathogen effectors are delivered into the host cell to suppress defense responses and MAPK modules are among the main targets, probably because of their central role in defense signaling (Feng and Zhou, 2012). As seen earlier, HopAI1 targets MPK3, MPK4, and MPK6 (Zhang et al., 2007, 2012). HopAI1 inactivates these MAPKs via its phosphothreonine lyase activity, which irreversibly removes the phosphate group from the threonine residue of the MAPK activation loop (Zhang et al., 2007). The *P. syringae* effector AvrB interacts with MPK4 and also induces the phosphorylation and activation of MPK4 in a manner promoted by the molecular chaperone HSP90 and its cochaperone RAR1 (Cui et al., 2010). In addition, MPK4 directly interacts with RIN4, a negative regulator of PTI (Afzal et al., 2011), and phosphorylates it *in vitro* (Cui et al., 2010). Overall, AvrB seems to activate the pathway formed by HSP90/RAR1-MPK4-RIN4 to perturb hormone signaling (e.g. induction of JA responses) and thus enhance host susceptibility (Cui et al., 2010). The *P. syringae* effector HopAO1 (formerly known as HopPtoD2) promotes pathogenicity and has *in vitro* protein tyrosine phosphatase activity (Bretz et al., 2003; Espinosa et al., 2003). A report suggested that HopAO1 can suppress PCD through inactivation of MAPK signaling, because transient overexpression of HopAO1 in tobacco suppressed the cell death induced by the constitutively active MAPKK NtMEK2 (Espinosa et al., 2003). However, it was then shown that HopAO1 was unable to block flg22-induced MPK3 and MPK6 activation in *Arabidopsis* protoplasts (He et al., 2006). A direct effect of HopAO1 on MAPKs is thus currently unclear. Concerning MAPKKs, the *P. syringae* effector HopF2 targets MKK5 and very probably other MAPKKs (MKK1, MKK3, MKK4, MKK6, and MKK10) (Wang et al., 2010b). Transient expression of HopF2 in protoplasts led to partial and total inhibition of MPK6 and MPK4 activity, respectively. Mechanistically, Wang et al. (2010b) demonstrated that HopF2 ADP-ribosylates MKK5 at R313, which blocks its kinase activity. Currently, no pathogen effectors are known to target *Arabidopsis* MAPKKKs. Besides direct inactivation of MAPK modules, some pathogen effectors also target upstream components of defense signaling, which notably inhibit MAPK modules. BIK1, PBS1, and PBLs are targets of the *P. syringae* effector AvrPphB, a cysteine protease that cleaves its substrates (Zhang et al., 2010). BIK1 and RIPK, a related protein, are targeted by the *Xanthomonas campestris* pv *campestris* effector AvrAC, which uridylylates and masks conserved phosphorylation sites in its substrates to prevent their activation (Feng et al., 2012). The *P. syringae* effectors AvrPto and AvrPtoB target the MAMP receptors FLS2, EFR, and CERK1 (He et al., 2006; Gohre et al., 2008; Xiang et al., 2008; Gimenez-Ibanez et al., 2009; Zeng et al., 2012). In addition, BAK1 is a convergent target of AvrPto, AvrPtoB, and HopF2 (Shan et al., 2008; Cheng et al., 2011; Wu et al., 2011; Zhou et al., 2014). Finally, Macho et al. (2014) showed that HopAO1 targets EFR, and potentially FLS2, and that both the direct physical interaction and the tyrosine phosphatase activity of HopAO1 contribute to the inhibition of elf18-induced activation of EFR and its downstream immune signaling. It is striking to see that most of the pathogen effectors described above act through multiple host targets and that a given immune component is often targeted by several different effectors, especially in the initial steps of the immune signaling.

SA, JA, ET, and Other Phytohormones

Phytohormones constitute another important class of signaling molecules involved in cell-to-cell coordination of responses. SA, JA, and ET are the major hormones implicated in plant innate immunity. SA signaling is usually involved against biotrophs or hemibiotrophs, while JA and ET signaling are generally important against necrotrophs (Glazebrook, 2005). SA, JA, and ET productions are triggered upon MAMP perception: SA production begins between 3 h and 6 h after flg22 treatment and reaches a peak after 9 h (a solution of 10 μM flg22 peptide was infiltrated into leaves of 4-week-old *Arabidopsis* Col-0 plants) (Tsuda et al., 2008), ET production starts around 1 h after flg22 treatment and peaks around 4 h (2-week-old *Arabidopsis* Col-0 seedlings were treated with 0.2 μM flg22) (Liu and Zhang, 2004), and JA production is, for example, elicited in potato (*Solanum tuberosum*) by Pep-13, an MAMP from *Phytophthora* species (lower leaves of wild-type potato plants were infiltrated with 100 μM Pep-13 and analyzed 24 h after treatment) (Halim et al., 2009). There is, however, no significant induction of JA production upon flg22 in *Arabidopsis* (Nomura et al., 2012). Using SA-deficient mutants (*sid2* and *pad4*), Tsuda et al. (2008) showed that SA contributes to flg22-triggered immunity against *P. syringae* and that disruption of SA signaling affects expression of MAMP-regulated genes. Some of the molecular mechanisms involved in MAMP control of phytohormone biosynthesis have been identified. Several works demonstrated that phosphorylation of ACS2 and ACS6 by MPK3 and MPK6 is a key step for ET production (Liu and Zhang, 2004; Han et al., 2010). Using the *dde2 ein2 pad4 sid2* quadruple mutant, Tsuda et al. (2009) showed that all single SA, JA, and ET signaling pathways contribute positively to flg22-triggered immunity and that PTI strongly depends on synergistic interactions between these signaling pathways.

Once SA, JA, and ET have been produced, these phytohormones are recognized by their receptors and convey different signaling and immune responses. For SA, some SA-binding proteins were identified, and a current model is that NPR3 and NPR4 are SA receptors that regulate NPR1 levels, leading to cell death or cell survival, according to SA concentrations (Yan and Dong, 2014). NPR1, although not clearly itself an SA receptor, is a master regulator of SA-mediated responses controlling gene expression (Mou et al., 2003; Wang et al., 2006), but some evidence also suggests that there are SA receptors other than NPR3 and NPR4 to mediate NPR1-independent pathways (Yan and Dong, 2014). The JA receptor is a complex made of coronatine-insensitive 1 (COI1) and jasmonate ZIM domain (JAZ) proteins and inositol pentakisphosphate (Xie et al., 1998; Katsir et al., 2008; Sheard et al., 2010). COI1 is an F-box protein that is part of the SCF^{COI1} ($Skp1/Cullin/F\text{-}box^{COI1}$) E3 ubiquitin ligase complex (Xu et al., 2002), and JAZ proteins repress MYC2, MYC3, and MYC4, key transcriptional activators of JA responses, by directly interacting with them (Chini et al., 2007; Fernandez-Calvo et al., 2011). JA induces the

COI1-mediated degradation of JAZ proteins by enhancing the interaction between COI1 and JAZs, leading to de-repression of JA-related transcriptional activation (Chini et al., 2007; Thines et al., 2007). Concerning ET, the understanding of its signaling has rapidly increased in the last few years, and the model has evolved from a linear cascade to a more complex pathway involving different feedback loops (Merchante et al., 2013).

In addition to SA, JA, and ET, other phytohormones were shown to play roles in plant immunity. Those are abscisic acid (ABA), brassinosteroids, gibberellins, cytokinin, and auxin, and their involvement in plant defense was recently reviewed (Ton et al., 2009; Kong et al., 2012b; Naseem and Dandekar, 2012; O'Brien and Benkova, 2013; De Bruyne et al., 2014). A picture is emerging whereby crosstalks occur between all these phytohormones, leading to modulation of plant susceptibility/resistance, with outcomes depending on the host genotype and the pathogen phylogeny (bacteria, fungi, etc.) and lifestyle (biotrophic, necrotrophic, etc.) (Pieterse et al., 2009; Robert-Seilaniantz et al., 2011; Naseem and Dandekar, 2012; Pieterse et al., 2012; Thaler et al., 2012; O'Brien and Benkova, 2013; De Bruyne et al., 2014).

Transcriptional Reprogramming

In plant immunity, transcriptional reprogramming can be considered as the main link between signal transduction (e.g. MAPK cascades) and implementation of induced defense mechanisms (e.g. production and secretion of antimicrobial compounds). It is a highly dynamic and controlled process involving numerous actors, notably transcription factors and chromatin regulators, and integrating multiple upstream immune signaling components (Moore et al., 2011).

MAMPs/DAMPs trigger a massive and dynamic reprogramming of plant genome expression. In *Arabidopsis*, several thousand genes are affected by flg22 (Denoux et al., 2008). The first transcriptional changes occur as early as 15 min after treatment (Nomura et al., 2012) and a part of these early gene modulations are independent of SA, JA, and ET signaling (Tsuda et al., 2009). After 1 h, the changes include notably genes involved in SA, JA, and ET signaling, synthesis of antimicrobial compounds and transcription regulatory factors, and at later times, genes notably involved in SA-mediated secretory processes and senescence (Denoux et al., 2008). We previously mentioned some precise elements of transcriptional regulation when we described known MAPK substrates. Several reports extensively reviewed the numerous transcription factors and also Mediator subunits known to be involved in plant immunity (Gutterson and Reuber, 2004; Eulgem and Somssich, 2007; Pandey and Somssich, 2009; Puranik et al., 2012; Alves et al., 2013; Ambawat et al., 2013; An and Mou, 2013; Gatz, 2013; Nuruzzaman et al., 2013; Buscaill and Rivas, 2014; Li et al., 2014a). Likewise, several reports demonstrated the contribution of chromatin remodelers/modifiers and chromatin modifications to defense transcriptional regulation (Ma et al., 2011; Berr et al., 2012; Dowen et al., 2012; Yu et al., 2012). Several studies reported links between MAMP-activated protein kinases, such as MAPKs and CDPKs, and transcriptional targets, notably showing synergistic or dominant effects between different protein kinases (Fiil et al., 2009; Boudsocq et al., 2010; Frei dit Frey et al., 2014). A comparative transcriptomic analysis of MPK3, MPK4, and MPK6 mutants recently showed that PTI is tightly choreographed by the integrate function of the three MAPKs, MPK3, MPK4, and MPK6 (Frei dit Frey et al., 2014). It became clear that MPK3 and MPK4 play an important role in repressing defense gene expression in the absence of MAMP stimulation. Moreover, the regulation of the large majority of MAPK targets occurs in a synergistic manner, requiring different combinations of the three MAPKs. The study also revealed that a defect in MPK3 or MPK6 affects the activities of the other MAPKs, indicating the existence of a complex feedback loop system between these three key regulators. Overall, these data show that plant immunity relies on fine-tuning a large gene network by the concerted action of multiple regulators.

PTI: Variations around a Single Theme

MAMPs/DAMPs trigger intracellular signaling events that may differ in time or intensity. Differences were observed in both lag phases and amplitudes of Ca^{2+} burst (Aslam et al., 2009; Ranf et al., 2011), extracellular pH (Zipfel et al., 2006), membrane depolarization (Krol et al., 2010), and ROS burst (Ranf et al., 2011). Kinase activities, notably of MPK3 and MPK6, showed differences, mainly in term of kinetics (Nuhse et al., 2000; Zipfel et al., 2006; Denoux et al., 2008). Transcriptome changes presented strong correlations, but some differences were observed in terms of the number of regulated genes, amplitude, and kinetics (Zipfel et al., 2006; Denoux et al., 2008; Ross et al., 2014). Ranf et al. (2011) could also show huge differences in Ca^{2+} burst between intact seedlings and isolated roots. These data indicate that MAMPs/DAMPs do not have the same immunogenic potential and that plant cells/tissues do not present a unique immune susceptibility.

CONCLUSIONS AND FUTURE PROSPECTS

Huge advances have been made to uncover the signaling network occurring during PTI. Yet, a lot of gaps within and between different signaling pathways have still to be filled in. For example, some data of Nomura et al. (2012) suggest a possible involvement of a MAPK cascade in the generation of Ca^{2+} signals, but no clear link has been established yet. Conversely, for instance, RBOHD is known to be connected to multiple signaling pathways (BIK1, CDPKs, PA, Ca^{2+}, etc.), and the challenge is now to better understand the effects of these inputs, whether they are positive, negative, synergistic, or antagonistic. Some signaling components in plant immunity have been identified in the last few years, such as CDPKs, 14-3-3 proteins, and heterotrimeric G proteins, and their integration in the defense signaling network has just started. The identification of many new signaling components will arise in part from screening for pathogen effector targets. Most of these effectors indeed target molecules involved in PTI signaling and protein–protein interaction studies recently identified numerous host candidates (Mukhtar et al., 2011; Wessling et al., 2014). In addition, protoplast systems as a medium-/high-throughput tool to identify effectors modulating PTI signal transduction should also contribute to the identification of new effectors targeting MAPK modules (Zheng et al., 2014). The identification of proteins interacting with

signaling components either by yeast two-hybrid or targeted approaches using purification of protein complexes will reveal not only novel players but certainly also connections between known signaling components. These approaches will also allow the identification of novel negative regulators of PTI signaling components, besides, for example, the known MAPK phosphatases (Bartels et al., 2010). Finally, signal-induced responses depend not only on the connections between the proteins in a network but also on reversible modifications of its components. Since protein kinases make up a very significant fraction of the components in the immune signaling systems, the study of the phosphorylation states of these components is obviously of major importance and should reveal the fine-tuning mechanisms in the plant innate immunity system.

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