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Microbial Signature-Triggered Plant Defense Responses and Early Signaling Mechanisms

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Abstract

It has long been observed that microbial elicitors can trigger various cellular responses in plants. Microbial elicitors have recently been referred to as pathogen or microbe-associated molecular patterns (PAMPs or MAMPs) and remarkable progress has been made on research of their corresponding receptors, signaling mechanisms and critical involvement in disease resistance. Plants also generate endogenous signals due to the damage or wounds caused by microbes. These signals were originally called endogenous elicitors and subsequently renamed damage-associated molecular patterns (DAMPs) that serve as warning signals for infections. The cellular responses induced by PAMPs and DAMPs include medium alkalization, ion fluxes across the membrane, reactive oxygen species (ROS) and ethylene production. They collectively contribute to plant pattern-triggered immunity (PTI) and play an important role in plant basal defense against a broad spectrum of microbial infections. In this review, we provide an update on multiple PTI responses and early signaling mechanisms and discuss its potential applications to improve crop disease resistance.

Keywords

Microbial elicitors; PAMPs/MAMPs: pathogen or microbe-associated molecular patterns; DAMPs: damage-associated molecular patterns; PTI: plant pattern-triggered immunity; signal transduction

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Introduction

In nature, plant resistance to microbial infections is the rule rather than the exception. Besides the preformed physical barriers, plants have evolved an innate immune system to recognize microbial invasion and launch effective defense responses to fend off pathogen attacks. As the first line of innate immune response, plant pattern-triggered immunity (PTI) was initiated upon perception of evolutionarily conserved microbial signatures, termed pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) [1,2]. PAMPs or MAMPs are often only present in the microbes and not the hosts. Endogenous damage-associated molecular patterns (DAMPs) are released from plants due to pathogen wounding or damage and serve as warning signals to trigger or amplify plant defense responses [3,4]. Perception of PAMPs/DAMPs is mediated through cell surface-resident pattern recognition receptors (PRRs), which are often encoded by receptor-like kinases (RLKs) or receptor-like proteins (RLPs) in plants [1,2]. Upon specific recognition of PAMP by the cognate PRR, the host elicits a series of cellular responses and physiological changes, such as a Ca^{2+} spike, extracellular alkalinization, membrane potential depolarization, ion effluxes, production of nitric oxide (NO), reactive oxygen species (ROS), and phosphatidic acid (PA), activation of an evolutionarily conserved MAP kinase (MPK) cascade, ethylene biosynthesis, callose deposition, and profound gene transcriptional reprogramming, which collectively results in plant resistance to pathogen attacks [1,2].

The signaling events that lead to these cellular and physiological responses in the plant have been a major focus of the studies in the plant-microbe interaction. Despite the detailed mechanisms that remain elusive; the framework underlying PAMP perception, signaling and responses is emerging. The understanding of PAMP-triggered plant basal resistance not only advances our general knowledge on the host immune signaling mechanisms but also holds significant promise to provide genetic resources and improve broad-spectrum and durable disease resistance in economically important crops. The yield loss caused by various diseases is one of the key challenges in crop production worldwide. In this review we summarize various physiological responses triggered by PAMPs/DAMPs and the recent advances in PTI signaling mechanisms.

Physiological responses triggered by PAMPs

As described in its original name, microbial elicitors, also called PAMPs, are the molecules from microbes capable of triggering plant defense responses [5]. The full repertoire of microbial elicitors remains unknown. Various PAMPs have been identified, such as the bacterial flagellin or its derived peptides flg22 and flgII-28, harpins, elongation factor Tu (EF-Tu) or its derived peptides elf18 and elf26, peptidoglycan (PGN), lipopolysaccharide (LPS), cold shock protein (CSP) and fungal chitin, Oomycete necrosis-inducing *Phytophthora* proteins (NPPs), cryptogein and elicitins [1]. Plant cell wall fragments or peptides derived from cleaved and degraded products, including oligogalacturonides (OG), prosystemin, hydroxyproline-rich systemins, proPeps and phytosulphokines, have been considered to be DAMPs [4,6]. Some PAMPs elicit responses in a wide range of plant species while others seem to be specific to certain plant species. Various distinct and overlapping physiological responses have been observed in diverse host systems depending

upon the different PAMPs perceived. A series of typical cellular responses have been established to serve as useful bioassays to monitor plant defenses upon PAMP perception. Some responses are initiated rapidly upon pathogen infection or elicitor treatment (within minutes) whereas some occur relatively late (within hours to days) [1].

Increase of Ca^{2+} concentration

A rapid increase of plant cell cytosolic Ca^{2+} concentration $[\text{Ca}^{2+}]_{\text{cyt}}$ in response to various PAMPs has been observed and represents an essential and common early event in PTI responses [7,8]. Treatment of parsley cells with the *Phytophthora sojae*-derived oligopeptide elicitor, Pep-13, induced a rapid increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ concentration within 4 minutes, which peaked at ~1mM and subsequently declined to sustained values of 300 nM. Interestingly, sustained increasing concentrations of $[\text{Ca}^{2+}]_{\text{cyt}}$ but not the transiently induced $[\text{Ca}^{2+}]_{\text{cyt}}$ are required for Pep-13-mediated activation of defense-associated responses [7]. Flg22 treatment induced a strong and rapid increase of $[\text{Ca}^{2+}]_{\text{cyt}}$ starting after a 30–40 sec lag phase and peaking after ~2–3 min, followed by a plateau phase of elevated $[\text{Ca}^{2+}]_{\text{cyt}}$, whereas PGN activated a much weaker and slower $[\text{Ca}^{2+}]_{\text{cyt}}$ increase [9]. Apparently, different PAMPs/DAMPs induce specific $[\text{Ca}^{2+}]_{\text{cyt}}$ elevations with flg22 having the highest $[\text{Ca}^{2+}]_{\text{cyt}}$ amplitude [8]. Nuclear Ca^{2+} concentration $[\text{Ca}^{2+}]_{\text{nuc}}$ is also elevated upon different PAMP treatments [10]. It appears that different PAMPs also induce specific spatial and temporal signatures of $[\text{Ca}^{2+}]_{\text{nuc}}$. Proteinaceous elicitors, including elicitins, flg22 and harpin, induced a pronounced and sustainable $[\text{Ca}^{2+}]_{\text{nuc}}$ elevation, whereas oligosaccharidic elicitors, such as the OG β -1,3-glucan laminarin induced little $[\text{Ca}^{2+}]_{\text{nuc}}$ elevation [10]. The significance of $[\text{Ca}^{2+}]_{\text{nuc}}$ rise and how it is perceived and transduced in plant defenses awaits to be elucidated in the future.

PAMP-induced cytosolic Ca^{2+} spike is most likely generated through two sources: the influx of extracellular Ca^{2+} and the release of Ca^{2+} from intracellular organelle stores, such as endoplasmic reticulum (ER) and vacuole [11]. Interestingly, the influx of extracellular Ca^{2+} , not intracellular Ca^{2+} , is essential for Pep-13-triggered immune responses as Pep-13-treated parsley cells maintained the normal defense responses in the presence of Ruthenium Red (RR) which inhibits Ca^{2+} release from intracellular compartments [7]. So far, the Ca^{2+} channels and how Ca^{2+} signals are sensed and transduced upon pathogen attacks or elicitor treatments still remain largely unknown. It has been suggested that cyclic nucleotide-gated channels (CNGCs) function in conducting Ca^{2+} to mediate plant immune responses [12,13]. There are three major types of Ca^{2+} sensors in plants, including calmodulin (CAM), calcineurin B-like proteins (CBLs) and calcium-dependent protein kinases (CDPKs) [14,15,16]. Recently, four *Arabidopsis* CDPKs (CDPK4, 5, 6 and 11) have been identified to play important roles, together with the MAPK cascades, in relaying primary flg22 and likely other PAMP signaling [17]. In addition to specific CDPKs, CAMs, CAM-like proteins (CMLs) and NO also mediate Ca^{2+} signaling and plant immune responses [12,13]. Future studies may identify other Ca^{2+} channels and elucidate the precise functions of Ca^{2+} sensors in mediating distinct and overlapping Ca^{2+} signatures triggered by different PAMPs.

Extracellular alkalization, membrane potential depolarization and ion fluxes

All plant cells have the capacity to maintain an electrochemical proton gradient across the plasma membrane (PM), generated by the PM-resident H^+ -ATPases, which pump H^+ from the cytosol to the extracellular space in an ATP-dependent fashion and maintain a negative membrane potential and a transmembrane pH gradient (acidic outside) [18]. The H^+ gradient plays an essential role in many physiological processes including ion uptake, solute transport, and cell wall growth [19]. Rapid and transient changes in extracellular or intracellular concentration of H^+ , often accompanied by PM potential depolarization, have been observed during various biotic and abiotic stress responses [19]. Medium alkalization due to altered ion fluxes across the plasma membrane, is one of the earliest responses observed in elicitor-treated plant cells. Bacterial flagellin and fungal chitin induce medium alkalization of *Arabidopsis*, tomato, tobacco and potato cell cultures within minutes [20]. A similar pattern but slightly weaker amplitude than flagellin was observed in EF-Tu-treated *Arabidopsis* cell cultures [21]. However, PGN induced a slower but more persistent increase in extracellular pH than flg22 [9]. In addition, Pep-13, harpin, cold shock protein, LPS and DAMP systemin have been shown to be able to elicit rapid and transient pH changes in different plant cell cultures [22,23,24]. By using different H^+ -ATPase inhibitors or activators, which promote or block extracellular alkalization in tomato cell culture, it has been suggested that the alkalization is positively correlated with systemin-induced genes [24]. Interestingly, treatment of fusicoccin, an activator of the H^+ -ATPases, which acidified the growth medium of tomato cell cultures, led to the accumulation of the plant defense hormone salicylic acid (SA) and the expression of pathogenesis-related genes. Apparently, the different PAMP or DAMP signaling pathways might be differentially regulated by changes in the proton electrochemical gradient across the plasma membrane [24].

Despite largely unknown mechanisms, several hypotheses, including activation of K^+/H^+ antiporters, H^+ /solute cotransporters, and other ion channels, as well as inhibition of the PM H^+ -ATPases, have been suggested to contribute to the extracellular alkalization [19]. The H^+ -ATPase activity has been considered as one of the mechanisms for PAMP-induced medium pH increase [25]. In many cases, PAMP-induced pH increase could be inhibited by Ca^{2+} blockers and protein kinase inhibitors, suggesting that Ca^{2+} signaling and protein phosphorylation are required for medium alkalization [24]. Consistently, phosphoproteomics suggested the phosphorylation dynamics of the *Arabidopsis* PM H^+ -ATPases upon flg22 treatment [26,27]. A significant reduction of phosphorylated peptides of H^+ -ATPases was observed in the samples treated with flg22, suggesting the dephosphorylation of H^+ -ATPases during PTI, which may explain the extracellular alkalization of media of cultured cells upon PAMP treatments.

Membrane depolarization and the flux of ions, including Cl^- , K^+ , and NO_3^- across the membrane, represent other classical and rapid responses of plant cells upon different PAMP treatments. Ion effluxes have been observed in various plant cells, such as parsley, tobacco, tomato, soybean and spruce [28,29,30,31]. Pharmacological experiments showed that ion fluxes could be inhibited by anion channel inhibitors, indicating that anion channels are involved in mediating the ion fluxes [29]. Using ion-selective microelectrodes, pronounced anion currents were recorded upon flg22 or elf18 treatment, suggesting that the signaling

cascades triggered by these two PAMPs converge on the same PM ion channels [30]. The kinase inhibitor K-252a blocked the ion efflux triggered by flg22, suggesting the involvement of protein phosphorylation in the activation of channels. This study also suggests that inhibition of the H⁺-ATPases is not the cause of proton influx, alkalization of the medium and depolarization. Flg22-induced membrane depolarization is associated with a cytosolic calcium rise, which might be a prerequisite for depolarization. However, ROS production might not be required for a membrane potential response since NADPH oxidase mutants were still depolarized upon elicitor stimulation [30]. Further studies are needed to elucidate the molecular nature of these early signaling events, including calcium influx, medium alkalization, ion flux and membrane depolarization in plant PTI.

Phosphatidic acid (PA) production

PA, a key intermediate of phospholipid biosynthesis has been proposed to convey a signaling function in plants [32]. A rapid and transient accumulation of PA and its phosphorylated derivative diacylglycerol pyrophosphate (DGPP) have been observed in tomato cells upon treatment with various PAMPs, including flg22, xylanase and chitin [33,34]. A rapid increase of PA and DGPP was also detected in tobacco cells treated with *Cladosporium fulvum* (Cf) fungal elicitor AVR4 that is recognized by receptor-like protein Cf4 [35]. In general, PA is generated by two different pathways: via activation of phospholipase D (PLD), which hydrolyzes structural phospholipids, and via activation of PLC, which generates diacylglycerol (DAG) that is phosphorylated to PA via DAG kinase (DGK) [32]. Interestingly, PLD-specific transphosphatidylation and differential ³²P-labelling assays indicate that AVR4-induced PA was mainly produced via the phosphorylation of DAG by DGK [35]. There are 7 DGK genes in *Arabidopsis*, and their potential involvement in plant defense awaits further genetic and biochemical investigations. PA generation is likely a prerequisite for ROS production since PLC inhibitors blocked the oxidative burst, which could be rescued by the application of PA [35]. Interestingly, plants displayed biphasic accumulation of PA in response to *Pseudomonas syringae* carrying either of two type III effectors, AvrRpm1 or AvrRpt2 that is recognized by intracellular plant resistance (R) protein for effector-triggered immunity (ETI) [36]. The first wave was correlated with the activation of PLC and DGK, which was similar with the pattern of elicitor-induced PA production. The second wave of PA accumulation was several orders of magnitude higher than the first wave, and was attributed to the activation of PLD. The data suggest the differential PA production and phospholipase activities in PTI and ETI signaling. The differential PA production is also coincident with the transient Ca²⁺ influx in PTI signaling and the prolonged Ca²⁺ influx in ETI signaling [37].

Reactive oxygen species (ROS) production

Production of ROS, including superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) has been observed upon infections with various pathogens or treatments with different PAMPs [38]. Similar as the patterns of Ca²⁺ influx and PA production, activation of plant R proteins elicited a biphasic ROS accumulation with a low-amplitude, transient first phase and a prolonged second phase of much higher magnitude. However, virulent pathogens only transiently induced ROS production [39], which was likely caused by the presence of multiple PAMPs in the pathogens. In addition to strengthening plant cell walls, ROS are

important signals to induce host defense responses, including gene transcriptional reprogramming [38]. Plant ROS are predominantly generated by plasma membrane-resident NADPH oxidases encoded by *RBOH* (Respiratory Burst Oxidase Homologue B) genes in plants. *Arabidopsis rbohD rbohF* double mutants showed decreased ROS production and cell death in response to *P. syringae avrRpm1* infection [40].

Recent advance has revealed the regulatory mechanisms of ROS production via NADPH oxidases. Chemical inhibitor analysis placed Ca^{2+} influx upstream of ROS production upon PAMP elicitation or pathogen infections [7,41]. Plant NADPH oxidase proteins contain two EF-hands in their N-terminus that could be regulated by Ca^{2+} signaling. Indeed, potato StCDPK4 and 5 directly phosphorylated and activated StRBOHB [42]. *Arabidopsis* CDPK1, 2, 4 and 11 strongly phosphorylated both RBOHD and RBOHF *in vitro* and mediated AvrRpm1-induced ROS production [37]. Mass spectrometric analysis revealed that *Arabidopsis* CDPK5 phosphorylated N-terminal serine residues of RBOHD *in vitro* and *in vivo*. The CDPK5 kinase activity was stimulated by flg22 or H_2O_2 treatment [43]. Consistent with this biochemical data, *Arabidopsis cpk5cpk6* double mutant displayed the reduced ROS production upon flg22 treatment [17]. Interestingly, rice CDPK12 seems to play a negative role in ROS production and the immunity to both virulent and avirulent blast fungus [44]. Differential phosphorylation of RBOH family members by CDPKs may account for this complex regulation. In addition to CDPKs, other kinases may also phosphorylate RBOH for ROS production in PTI signaling.

Callose deposition

Callose is a plant polysaccharide composed of β -1, 3-glucan polymer, which serves as a matrix for antimicrobial compound deposition in plant cells [45,46]. Upon pathogen infection, callose is deposited in cell wall appositions, called Papillae, at the sites of attack to form effective chemical and physical defense barriers for pathogen invasion. Purified PAMPs, including flg22, elf18, chitin and DAMPs, including OG, have been shown to induce callose deposits in leaves or cotyledons of *Arabidopsis*, which has emerged as an indicator of plant immune responses [46]. Callose is synthesized at the cell wall by callose synthases with UDP-glucose as a substrate [45]. Interestingly, a callose synthase mutant, *pmr4*, which lost the induced callose response to fungal infection, became resistant, rather than susceptible to pathogens. The enhanced resistance is likely due to the elevated defense hormone salicylic acid (SA) signaling pathway, suggesting that callose or callose synthase negatively regulates the SA pathway [47].

The pathogen-induced callose deposits are regulated by intrinsic and extrinsic cues. Plant growth and environmental conditions, including light and humidity, have impact on the intensity of callose deposits. ROS positively regulate flg22- and OG-induced callose [48]. Interestingly, the plant hormone abscisic acid (ABA) could have either a positive or negative effect on callose deposition depending on plant growth conditions [46]. Different PAMPs may employ different mechanisms to induce callose. For example, flg22-induced callose was correlated with RBOHD-produced H_2O_2 , whereas chitosan induced callose independently of ROS production. In addition, flg22-induced callose completely depended on PMR4, whereas approximately 10% of chitosan-induced callose came from PMR4-

independent pathways [46]. Thus, callose deposition is a complex defense response that is influenced by multiple signals and regulated by different signaling pathways. Biosynthesis of 4-methoxylated indole glucosinolate, a secondary metabolite implicated in preventing damage by herbivores, was shown to be required for flg22-mediated callose deposition and functions as a plant defense component against bacterial and fungal pathogens [49,50]. The studies also suggest the positive correlation between pathogen-triggered callose deposition and resistance to pathogen infections since genes necessary for pathogen resistance are also required for both callose deposition and glucosinolate activation [50].

In addition to the above mentioned physiological responses, several other assays have been used to quantify the activity of immune responses. Perception of various PAMPs induces ethylene production in different plants [1]. The biosynthesis of camalexin, the major phytoalexin in *Arabidopsis*, has been found to be induced by flg22 [51]. Stomata are utilized as one of the important entry routes for pathogens to enter apoplasts [52]. Rather than serving as passive ports for pathogen entry, stomata actively respond to pathogen infections [53]. Purified PAMPs, including flg22 and LPS, or non-pathogenic bacteria caused rapid stomatal closure in *Arabidopsis* possibly as a mechanism to avoid bacterial invasion [53]. The treatment with flg22 or elf18 suppressed *Arabidopsis* seedling growth which is likely caused by the prolonged and collective activation of PTI responses [1]. Despite an unclear mechanism, flg22-mediated seedling growth inhibition has been successfully used to identify the cognate FLS2 receptor [54]. Pre-induction of PTI responses could suppress the cell death triggered by type III effectors translocated into plant cells, which has been used to identify genes involved in PTI signaling in tomato [55]. Although many plant defense responses are considered cell autonomous, treatment of PAMPs, such as flg22 and chitin caused a reduction in molecular flux via plasmodesmata, which is independent of the known intracellular signaling pathways [56]. The study not only provides an uncharacterized PAMP response marker, it also suggests the importance of cell-to-cell communication in plant defenses.

Early signaling mechanisms in PTI

PAMPs/DAMPs are usually perceived by PM-localized RLKs or RLPs in plants. So far, more than a dozen PAMP/DAMP receptors have been identified in different plant species [2]. Some of the best studied PAMP receptors are *Arabidopsis* FLS2 recognizing flg22, EFR recognizing elf18, CERK1 recognizing chitin, PERP1/2 recognizing DAMP Pep1, and rice Xa21. Although perceived by specific receptors, different PAMPs appear to trigger the convergent downstream immune signaling events, including receptor dimerization, activation of receptor-like cytoplasmic kinases (RLCKs), MAPKs and CDPKs, which govern downstream immune gene reprogramming (Fig. 1). Plant immune responses are also subject to negative regulation by phosphatases and ubiquitination-mediated protein degradation to avoid over-activation of defense responses.

Dimerization of receptor-like kinases

Receptor dimerization plays an essential role in triggering intracellular signaling. In animal innate immunity, the PAMP receptors TLR1 and TLR2 heterodimerize upon stimulation with a triacylated lipopeptide to initiate downstream signaling [57]. Upon flg22 perception,

FLS2 instantaneously associates with another PM-localized RLK BAK1 (BAK1), thereby initiating downstream signaling [58,59] (Fig. 1). BAK1 is required for signaling triggered by multiple PAMPs, including flg22, elf18, harpin, LPS, PGN, oomycete elicitor INF1 and bacterial cold-shock protein in *Arabidopsis* and *Nicotiana benthamiana* [58,59,60]. Consistent with this genetic requirement, BAK1 has been shown to heterodimerize with several PAMP/DAMP receptors including FLS2, EFR, and PEPR1/2 [61,62]. BAK1 belongs to a subfamily of somatic embryogenesis receptor kinases (SERK) involved in plant immunity and development [62,63]. Its closest homolog SERK4 or BKK1 play a redundant role with BAK1 in plant immunity and cell death control [62,65]. Although it was shown that BAK1 was not required for flg22 binding to FLS2, the recent crystal structure of flg22-FLS2-BAK1 complex revealed that BAK1 functions as a co-receptor by recognizing the C terminus of the FLS2-bound flg22 [58,66]. The similar mechanism might be used for BAK1 to complex with other PAMP receptors.

BAK1 is not required for fungal chitin signaling and does not interact with chitin receptor CERK1 [67]. The crystal structure of chitin and the lysine motif (LysM)-containing ectodomain of CERK1 indicates that chitin induces AtCERK1 homo-dimerization which is critical for CERK1 activation and signaling [68]. The homo-dimerization or conformational changes of the leucine-rich repeat (LRR)-containing ectodomain of FLS2 was not observed in the flg22-FLS2-BAK1 complex [66]. Although chitin could bind to CERK1, it displayed a relatively low chitin-binding affinity *in vitro* [69,70]. In rice, a PM-resident glycoprotein (CEBiP) with two extracellular LysM motifs was shown to directly bind to chitin [71]. Rice OsCERK1 is also required for chitin responses and forms a complex with CEBiP upon chitin recognition [72]. The role of *Arabidopsis* CEBiP-like genes *LYM* in chitin perception and signaling is very unique. There are three *LYM* genes in the *Arabidopsis* genome and *LYM2* was identified in a chitin pull-down assays, suggesting that it either binds to chitin itself or is a part of chitin receptor complex [70]. Interestingly, *LYM2* is required for chitin-induced and CERK1-independent reduction in molecular flux via plasmodesmata, but not for CERK1 activity and CERK1-mediated ROS production and MAPK activation [56]. This study suggests that there are at least two independent chitin perception and activation systems in *Arabidopsis*. The differential role of *LYM* and CERK1 in *Arabidopsis* chitin signaling was further confirmed by that knockout of all three *Arabidopsis LYM* genes did not compromise CERK1-mediated chitin responses. However, a CERK1 family member, *LYK4*, is required for chitin-induced responses in *Arabidopsis* [73]. It remains unknown whether CERK1 and *LYK4* form a complex in recognizing chitin. Interestingly, *LYM1* and *LYM2* bind directly to PGN *in vitro*, which is structurally related to chitin. Together with CERK1, *LYM1* and *LYM2* are required for PGN-mediated responses and immunity to bacterial infections, suggesting that these three LysM domain proteins are part of a plant PGN receptor complex [74].

Activation of receptor-like cytoplasmic kinases (RLCKs)

Similar to the case of RLKs, plants have also evolved a significant number of RLCKs that often functionally and/or physically associate with RLKs to relay intracellular signaling via transphosphorylation events [75]. The Botrytis-induced kinase 1 (BIK1), a PM-localized RLCK, is rapidly phosphorylated upon flagellin perception in an FLS2- and BAK1-

dependent manner [76,77]. BIK1 interacts with FLS2 and BAK1 and is directly phosphorylated by BAK1. Upon flg22 perception, BIK1 is released from the FLS2/BAK1 complex to transduce intracellular signaling likely due to its phosphorylation by BAK1 [76,77,78]. Consistent with the broad role of BAK1 in plant immunity, BIK1 is also involved in elf18-mediated immune responses and interacts with EFR [77]. In addition, BIK1 interacts with the DAMP Pep1 receptor PEPR1 and is phosphorylated by PEPR1 *in vitro* [79]. It is likely that BIK1-mediated Peps/PEPRs signaling acts downstream or in parallel with ethylene signaling pathways to amplify plant PAMP responses [79,80]. In addition to bacterial resistance, BIK1 is also an important player for plant resistance to necrotrophic fungi, *B. cinerea* and *Alternaria brassicicola*, likely via jasmonate and ethylene-regulated signaling pathways [81]. BSK1, a RLCK involved in plant steroid hormone Brassinosteroid signaling, was recently found to physically associate with FLS2 and regulate plant innate immunity [82]. Similar with BIK1, BSK1 is released from FLS2 upon flg22 perception, suggesting that BSK1 may use the similar mechanism with BIK1 in mediating FLS2 signaling [82]. Identification of BIK1 phosphorylation sites and substrates will elucidate how BIK1 activity is regulated by the PAMP receptor complex and how BIK1 transduces intracellular signaling to activate plant defenses.

It still remains unknown whether BIK1 functions upstream of MAPK cascades and/or CDPKs in *Arabidopsis* flagellin signaling. A rice RLCK, OsRLCK185, was identified as a target of *Xanthomonas oryzae* type III effector Xoo1488, which suppresses both PGN and chitin-induced responses [83]. OsRLCK185 is required for chitin and PGN-mediated MAPK activation and defense gene expression, suggesting that OsRLCK185 functions upstream of the MAPK cascade. Consistently, overexpression of OsRLCK185 enhanced chitin-induced MAPK activation. Similar with BIK1, OsRLCK185 associates with, and is directly phosphorylated by OsCERK1 at the plasma membrane upon chitin perception [83]. It will be interesting to test whether OsRLCK185 directly regulates MAPK activation through phosphorylation. It has been suggested that the RLCK protein SSP controls the embryonic patterning process by activation of the MAPK cascade [84].

Activation of MAPK and CDPK

A MAPK signaling cascade generally involves three functionally tiered protein kinases, a MAP kinase (MAPK), a MAPK kinase (MAPKK/MEK/MKK), and a MAPK kinase kinase (MAPKKK/MEKK), that transduce and amplify extracellular and intracellular stimuli into a wide range of overlapping or specific intracellular responses in eukaryotic cells [85]. MAPK activation is one of the early signaling events following PAMP recognition in both plants and animals [86,87,888]. To date, several MAPKs, including MPK3, MPK4, MPK6 and MPK11, have been shown to be activated by flg22 and other PAMP treatments [89]. Accumulating evidence suggests that the perception of PAMPs activates two branches of MAPK cascades in *Arabidopsis*, MEKK1/MEKKs-MKK4/5-MPK3/6 and MEKK1-MKK1/2-MPK4 [88] (Fig. 1). It is believed that the MAPK cascade regulates plant immunity through activation of defense-related genes via direct phosphorylation of downstream transcription factors, such as WRKYs and ERFs [89]. Activation of *Arabidopsis* MPK3/6 or their tobacco orthologs induces some defense-related genes. It has also been observed that phosphorylation of ERF6 or ERF104 by MPK3 and/or MPK6

activates the expression of pathogenesis-related and defensin genes, such as *PDF1.1*, *PDF1.2a* and *PDF1.2b* [90,91]. Although MAPK activation is a robust and potent immune marker by multiple PAMP treatments, genetic evidence of their role in plant immunity is lagging behind their biochemical functions because of gene redundancy and the lethality of certain mutants.

As mentioned above, the rapid increase of cytosolic Ca^{2+} concentration has been observed in the plants response to PAMPs or pathogen effectors. CDPKs, as a class of important Ca^{2+} sensors, have been implicated in plant immunity. CDPKs function as Ca^{2+} sensor protein kinases with both a protein kinase domain and a calmodulin-like domain with four EF hands for Ca^{2+} binding [14,15]. Tobacco NtCDPK2 was activated by elicitor Avr9 recognized by RLP Cf-9 and is required for Cf-9-mediated cell death in *Nicotiana benthamiana* [92]. In *Arabidopsis*, overexpression of AtCDPK1 confers broad-spectrum resistance to both bacteria and fungi [93]. CDPKs function in PTI signaling mainly through controlling transcriptional reprogramming of immune genes and ROS production via phosphorylation of RBOH family members (see above). Ca^{2+} channel blockers La^{3+} and Gd^{3+} diminished flg22-induced defense gene expression, whereas ectopic expression of active AtCDPK5 or AtCDPK11 induced a set of largely overlapping genes with flg22 and other PAMP-induced early genes [17]. Transient expression of active NtCDPK2 triggered the accumulation of JA and ET, but not SA, accompanied by the activation of JA- and ET-regulated genes [94]. A recent study also suggests that *Arabidopsis* CDPK5 functions through enhanced SA production and signaling [43].

Ubiquitination-mediated protein degradation

An early and general response following the activation of cell-surface receptors is the receptor endocytosis and subsequent intracellular degradation. This down-regulation of immune receptors provides a mechanism to prevent excessive or prolonged activation of immune responses. A major mechanism for receptor degradation is the ubiquitination of the cytosolic domain of cell-surface receptors [95]. Ubiquitination is a posttranslational modification that directs covalent conjugation of conserved ubiquitin molecules to specific protein substrates through a stepwise reaction mediated by three distinct classes of enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3) [96]. The substrate specificity is dictated by ubiquitin E3 ligases. Some E3 ligases have been implicated in plant innate immunity. Two closely related plant U-box E3 ubiquitin ligases, PUB12 and PUB13, ubiquitinate FLS2 and lead FLS2 degradation [97]. PUB12 and PUB13 interact with BAK1, and are directly phosphorylated by BAK1. Upon phosphorylation, PUB12 and PUB13 are recruited to FLS2 and polyubiquitinate FLS2 [97]. Thus, PUB12 and PUB13 negatively regulate plant innate immunity by down-regulating PAMP receptor stability. PUB22, PUB23 and PUB24, another subgroup of *Arabidopsis* U-box E3 ligases, also negatively regulate flagellin-mediated signaling [98]. Instead of ubiquitinating FLS2, PUB22 interacts with and ubiquitinates Exo70B2, a subunit of the exocyst complex that mediates vesicle tethering during exocytosis and plays a positive role in multiple PAMP-triggered responses [99]. Not all the E3 ligases negatively regulate plant immune responses. Two U-box E3 ligases, ACRE276 and ACRE74 are required for Avr9-Cf9-triggered cell death and disease resistance [100]. A RING-type E3 ubiquitin ligase,

XB3, interacts with the kinase domain of rice RLK XA21 that confers resistance to specific races of *Xanthomonas oryzae* pv. *oryzae* (Xoo) [101]. XB3 is directly phosphorylated by XA21 and positively regulates XA21 signaling. Although the substrates for ACRE276, ACRE74 and XB3 are unknown, it is likely that they degrade certain negative regulators in plant disease resistance.

In addition to the above components, other signaling molecules are also involved in plant immune responses. Some phosphatases could negatively regulate plant immunity by dephosphorylating important PAMP sensing and signaling components. A protein phosphatase 2C (PP2C), XB15 negatively regulates the XA21-mediated innate immune response by dephosphorylating XA21 [102]. *Arabidopsis* MAPK phosphatase 1, MKP1, negatively regulates multiple PAMP responses and resistance to *P. syringae* [103]. Rice small GTPase OsRac1 and guanine nucleotide exchange factor OsRacGEF1 were found in rice OsCERK1/OsCEBiP-mediated “defensome” and are involved in chitin-induced immunity [104]. Heterotrimeric G proteins could also function as a convergent point in mediating defenses activated by multiple RLKs [105].

Concluding remarks and perspectives

Great strides have been made over the past decade towards characterizing plant immune responses and dissecting the underlying molecular mechanisms. But many questions that are central to our understanding of PAMP perception and signaling activation still remain. It remains unknown how RLK/RLCK complexes activate the MAPK/CDPK signaling cascade. Do RLKs/RLCKs directly phosphorylate MAPKKKs to relay the signal or other components, such as small GTPase or are heterotrimeric G proteins involved in MAPK activation? Heterotrimeric G protein complex is required for flg22-induced MPK4, but not MPK3 and 6 activation [105]. Recent studies also suggest that maize heterotrimeric G α protein functions in RLK CLAVATA signaling to control shoot meristem size through interaction with RLP CLAVATA2 [106]. We also do not know how these early signaling events regulate immune gene transcriptional reprogramming. The essential transcription factors downstream of MAPKs and CDPKs in PTI signaling have not been identified. A sensitive genetic screen aiming at immune gene transcriptional regulation may uncover some novel components in plant immunity. Alternatively, MAPKs or CDPKs may regulate other effector proteins in the control of gene expression. Ultimately, we aim to apply the knowledge learnt from model plants to improve crop plant disease resistance. Since PAMPs are conserved microbial signatures, PAMP-triggered immunity is considered broad spectrum against bacterial, fungal and virus infections. EFR is only found in the members of the *Brassicaceae*. Expression of EFR in the *Solanaceous* plants tomato and tobacco rendered plants more resistant to a range of phytopathogenic bacteria from different genera. This provides an opportunity to engineer EFR in different plant families to achieve durable and sustainable resistance [107]. Similarly, barley R protein MLA1 is fully functional in *Arabidopsis* against the powdery mildew fungus, suggesting ~200 million years of evolutionary conservation of the underlying immune mechanism [108]. Better understanding the origin and evolution of plant immune systems by comparing different lower and higher plants will be essential for interfamily transfer of immune components for broad-spectrum or race specific resistance.

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Highlights

- Microbial signatures induce a series of plant immune responses
- Different microbial signatures are perceived by specific plant receptors
- Diverse microbial signatures induce convergent signaling mechanisms
- Plant pattern-triggered immunity provides basal resistance to pathogen infections
- Understanding pattern-triggered immunity provides insights into improving broad-spectrum resistance in plants

Figure 1.

A Model of flagellin-triggered immune responses and signal transduction pathways. A. The resting state of FLS2 and BAK1 at the plasma membrane without microbial infection. In the absence of microbial elicitor flagellin, FLS2 and BAK1 may not form a stable complex. BAK1 is associated with BIK1 and PUB12/13 and FLS2 is also associated with BIK1. B. flagellin-triggered immune responses and signal transduction pathways. Flagellin perception induces FLS2 and BAK1 association and phosphorylation. Activated BAK1 phosphorylates BIK1, which in turn transphosphorylates the FLS2/BAK1 complex. Phosphorylated BIK1 is released from the FLS2/BAK1 complex. No direct phosphorylation targets of FLS2 have yet been identified. FLS2 and BAK1 association also recruits PUB12/13 into the receptor complex. BAK1 directly phosphorylates PUB12/13 which in turn ubiquitinates FLS2 leading to FLS2 degradation and down-regulating FLS2 signaling. Activation of receptor complex leads to the activation of Ca^{2+} flux through Ca^{2+} channel, Cl^- efflux and H^+/K^+ movement across the plasma membrane. MAPK and CDPK cascades are initiated downstream of the activated receptor complex and further mediate the defense gene expression.