Chapter 18

Signalling Crosstalk of Plant Defence Responses to Xylem-invading Pathogens

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Additional information is available at the end of the chapter

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Abstract

Xylem is a plant vascular tissue that transports water and dissolved minerals from the roots to the rest of the plant. It consists of specialized water-conducting tracheary elements, supporting fibre cells and storage parenchyma cells. Certain plant pathogenic fungi, oomycetes and bacteria have evolved strategies to invade xylem vessels and cause highly destructive vascular wilt diseases that affect the crop production and forest ecosystems worldwide. In this chapter, we consider the molecular mechanisms of root-specific defence responses against vascular wilt pathogens, with an emphasis on the most important and well-studied fungal (*Verticillium* spp. and *Fusarium oxysporum*) and bacterial (*Xanthomonas* spp. and *Ralstonia solanacearum*) pathogens. In particular, we present the current understanding of plant immune responses, from invasion perception to signal transduction and termination. Furthermore, we address the role of specific transcription factors involved in plant immunity and their regulatory network. We also highlight the crucial roles of phytohormones as signalling molecules in local and systemic defence responses. Finally, we summarize the current knowledge of plant defence responses to xylem-invading pathogens to devise new strategies and methods for controlling these destructive plant pathogens.

Keywords: Vascular wilt pathogens, effectors, plant innate immunity, signal transduction, biotic stress

1. Introduction

The disease triangle concept, introduced in the 1960s by George McNew to predict plant disease outcomes, shows the complex interactions among the environment, the host and the infectious (or abiotic) agent [1]. Plants, continuously challenged by numerous abiotic stresses, potential pests and pathogens, have evolved efficient strategies to perceive and respond to

such threats. Plants lack specialized immune cells and their survival relies upon a highly sophisticated innate immune system, in which each plant cell responds autonomously [2–5]. The first line of defence is a basal resistance response called pattern-triggered immunity (PTI). It is induced by recognition of exogenous microbe or pathogen-associated molecular patterns (MAMPs or PAMPs) or endogenous molecules released on pathogen perception or pathogen-induced cell damage (damage-associated molecular patterns, DAMPs) via pattern-recognition receptors (PRRs) in the plasma membrane [4]. Successful pathogens overcome PTI by secreting effectors, hydrolytic enzymes or toxins, which suppress or interfere with host defence molecules [6]. In an evolutionary arms race, plants have evolved a robust defence response network termed effector-triggered immunity (ETI) to intercept pathogen effectors through intracellular receptors, such as nucleotide-binding site/leucine-rich repeat (NLR) proteins [5,7,8]. An intricate network of signalling pathways transduces these incoming signals into a diverse array of immune responses activating reactive oxygen species (ROS) generation, MAP kinases, Ca^{2+} signalling, the production of phytohormones and extensive transcriptional reprogramming [9].

In the past, comprehensive research has been dedicated to understanding plant physiological and molecular responses to individual abiotic and biotic stresses under controlled laboratory conditions. Recent studies of plant responses to concurrent abiotic and biotic stress conditions [10–16] have demonstrated that plants perceive and respond to combined stresses in a specific and unique manner. Moreover, the underlying signalling pathways are carefully modulated [14,17,18] and coordinated to ensure that plant growth and fitness are not significantly retarded [19].

Vascular wilt pathogens are soil-borne bacteria, fungi and oomycetes that employ various infection strategies to invade plant roots at different infection sites [20]. They subsequently advance inter- or intracellularly through the root cortex and enter the xylem vessels, where they proliferate and spread passively with xylem sap to aerial plant parts [21]. The characteristic wilt symptoms develop as a consequence of obstructed transportation of water and minerals, either due to the physical blockage of vessels by the pathogen or indirectly due to the activation of plant physical defence responses (e.g. formation of tyloses, accumulation of pectin-rich gels and gums) that confine the further spread of the pathogen [22]. In addition to wilting, other disease symptoms include vein clearing, leaf epinasty, chlorosis, vascular browning, stunting, necrosis and eventually plant death [21–24].

Primarily due to the specific lifestyle of vascular wilt pathogens, relatively little is known about their interactions with host plants and root-specific defence responses on molecular and biochemical levels compared to foliar pathogens. This chapter, therefore, summarizes the currently available molecular, cellular and systems biology data gathered from studies of signalling networks in model plants and crops challenged by bacterial or fungal pathogens and applies this general knowledge to advance understanding of vascular wilt pathogenesis and implement all these findings into the design of new strategies for the protection of crops and forest ecosystems.

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2. Plant immune signalling initiation

Several factors contribute to the complex regulatory mechanisms in the initiation of plant immune signalling: (i) as sessile organisms, plants need to respond promptly to danger signals, (ii) each plant cell reacts autonomously to different stimuli, but the response needs to be integrated at a higher organizational level to ensure the plant's survival and (iii) immune reactions are energy- and resource-demanding processes requiring the proper timing and amplitude of response [4]. Typically, immune responses occur on recognition of conserved microbe-, pathogen- or damage-associated molecular patterns or after perception of effector molecules that are species-, race-, or strain-specific and contribute to pathogen virulence [25]. However, not all microbial elicitors conform to the common distinction between PAMPs and effectors, and so Thomma et al. [26] proposed that plant immunity should be considered as a continuum, instead of a two-branched system composed of PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). An alternative perspective of plant innate immunity as a system that evolves to detect invasion has recently been extended into a so-called invasion model [27]. In this model, invasion patterns (IPs), externally encoded or modified-self ligands that signify invasion, are perceived by plant invasion pattern receptors (IPRs) and induce IP-triggered responses that do not result in immunity by default.

2.1. Plant PRRs convey danger signals to the intracellular immune signalling pathways

Recently emerging structural biology data on plant–pathogen interactions, [28] together with data obtained primarily from genetic and biochemical studies on the leaves of model plants and crops, have revealed that fine-tuning and coordination of immune responses are achieved within large protein complexes at the plasma membrane, where plant PRRs reside [4]. Plant PRRs are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs) [4,6,25,29]. RLKs are modular proteins comprised of an extracellular domain involved in recognition of MAMPs/DAMPs, a single-pass transmembrane domain and a cytosolic serine/threonine kinase domain that transmits a signal to downstream signalling components. RLPs have a similar architecture, but their short cytoplasmic tail lacks kinase activity. RLPs, therefore, probably form heteromeric complexes with RLKs or other cytosolic kinases to relay downstream signalling. Extracellular domains of PRRs contain various motifs involved in recognition and binding of ligands. Leucine-rich repeat (LRR) motifs are widespread and serve as a scaffold for protein- or peptide–protein interactions [30]. Well-characterized examples of LRR–RLKs are *Arabidopsis* FLS2 (flagellin-sensitive 2) and EFR (elongation factor Tu receptor), which bind flagellin fragment flg22 and EF-Tu peptide elf18, respectively [31,32]. Lysine motifs (LysMs), lectin and epidermal growth factor (EGF)-like domains are found in PRRs that recognize carbohydrate moieties, such as fungal chitin [33,34] or bacterial peptidoglycans [35].

2.1.1. Perception of chitin

The chitin-responsive PRR system has been thoroughly investigated in both dicots and monocots. The perception of chitin in monocot plants is best described in rice (*Oryza sativa*). Chitin elicitor binding protein (OsCEBiP) is a receptor-like protein that specifically binds chitin

oligomers [36]. In the absence of chitin, OsCEBiP exists as a homodimer [37]. On binding of chitin octamer, OsCEBiP associates with receptor-like kinase OsCERK1 and forms heterodimers [37,38]. This interaction activates the OsCERK1 kinase domain to become phosphorylated. Subsequently, active OsCERK1 phosphorylates a guanine nucleotide exchange factor OsRac-GEF, which activates a Rho-type small GTPase OsRac [39]. OsRac acts as a molecular switch in many plant signalling pathways and, among other things, regulates the production of ROS by the NADPH oxidase OsRbohB [40].

The model dicot plant *Arabidopsis thaliana* harbours chitin elicitor receptor kinase 1/LysM-containing receptor-like kinase1 (CERK1/LYK1), which perceives chitin (a polymer of N-acetyl-D-glucosamine, NAG) through its LysM motif [33]. Although the AtCERK1 extracellular domain contains three tandem LysMs, only LysM2 binds NAG_5 [41]. This interaction, however, fails to trigger immune responses. Downstream signalling has been observed only on binding of chitin octamer, which acts as a bivalent ligand and induces CERK1 dimerization [41]. Another LysM-containing cell surface receptor, AtLYK5, has recently been proposed as the primary chitin receptor, due to a significantly higher binding affinity for NAG_8 compared to AtCERK1 [42]. AtLYK5 exists as a homodimer in the absence of chitin. Binding of chitin to AtLYK5 homodimer promotes the association of AtLYK5 with AtCERK1. This leads to dimerization of AtCERK1 and activation of its kinase domain. The chitin signal is then transduced downstream to mitogen-activated protein kinases MPK3 and MPK6 [33].

2.1.2. Perception of flagellin

Extensive research of the model plant *Arabidopsis thaliana* has elucidated molecular mechanisms triggered in response to recognition of bacterial flagellin by evolutionary conserved LRR RLK flagellin-sensitive 2 (FLS2) [31]. The extracellular domain of FLS2 contains 28 LRR and binds the 22-amino acid long flagellin epitope flg22 [43]. Immediately after, FLS2 associates with co-receptor brassinosteroid insensitive 1 (BRI1)-associated receptor kinase 1 (BAK1)/somatic embryogenesis receptor-like kinase 3 (SERK3) to form a heterodimer [44–46]. BAK1 is a key regulatory LRR RLK coordinating growth–defence trade-offs [47], since it is required for early defence responses in PTI [48] but also implicated in brassinosteroid hormone signalling [49]. BAK1 phosphorylates receptor-like cytoplasmic kinase *Botrytis*-induced kinase 1 (BIK1), which interacts and forms a complex with both BAK1 and FLS2 [50]. BIK1 is subsequently auto-phosphorylated at tyrosine and serine/threonine residues [51]. Activated BIK1 contributes to flg22-triggered calcium influx from apoplast [52] and phosphorylates NADPH oxidase RbohD involved in reactive oxygen species (ROS) production [53].

2.1.3. Perception of peptidoglycan

Peptidoglycan (PGN), a polymer of N-acetylglucosamine and N-acetylmuramic acid branched with a short peptide, is an essential component of a bacterial cell wall and is another well-studied MAMP. In *Arabidopsis,* the PGN perception system is comprised of two GPI-anchored LysM domain RLPs, LYM1 and LYM3, which bind PGN, and a transmembrane RLK AtCERK1, which probably relays the PGN signal downstream [35]. Rice has a similar PGN detection

system, involving LysM RLK OsCERK1 [54] and two LysM-containing proteins, OsLYP4 and OsLYP6, which not only bind PGN but also associate with chitin oligomers [55].

2.1.4. Perception of DAMPs

Some PRRs respond to damage-associated molecular patterns (DAMPs), endogenous molecules such as cell wall fragments or peptides released on pathogen attack or various abiotic stresses [25,56]. Partial degradation of pectic polysaccharide homogalacturonan (HGA) by pathogen or plant polygalacturonases produces oligogalacturonides (OGs), oligomers of α-1,4–linked galacturonic acid [57,58]. In *Arabidopsis*, OGs are perceived by wall-associated kinase 1 (WAK1) Ser/Thr RLK kinase with an extracytoplasmic domain that contains several EGF-like repeats [59]. The signal is then relayed to the MAP kinase signalling pathway, where AtMPK3 and AtMPK6 become phosphorylated and induce expression of several defence genes [60]. Additional OGs-triggered defence responses include activation of NADPH oxidase AtRbohD involved in the generation of ROS, production of NO and deposition of callose in the plant cell walls [57,58].

The best studied peptides acting as DAMPs belong to the plant elicitor peptides (Peps) family. They are processed from precursor PROPEPs [61,62]. AtPep1, a 23 amino acid peptide released from the C-terminal of PROPEP1, was the first peptide elicitor isolated from *Arabidopsis* [61], but similar peptides were also later confirmed in other plants [63]. AtPep1 is recognized by two LRR RLK, PEPR1 and its paralog PEPR2 [64,65]. However, signalling is initiated only in complex with co-receptor LRR RLK BAK1 [66]. The active receptor complex consequently induces the expression of MAP kinase 3 (MPK3), WRKY transcription factors and defence-related genes such as *PR-1* and *PDF1.2* (encoding defensin) [63]. Moreover, the cytosolic kinase domain of PEPR1 has guanylyl cyclase activity, which generates cGMP from GTP [67]. An increased local concentration of cGMP has been proposed to open cyclic nucleotide-gated channels (CNGC2) in the plasma membrane and activate cytosolic Ca^{2+} signalling [67,68].

2.2. Intracellular immune receptors NLRs detect pathogen effectors

In addition to PRRs, plants have evolved a second class of immune receptors that intercept effectors in different parts of the cell [8]. These intracellular receptors, so called NLRs, are characterized as multi-domain proteins that have a conserved central nucleotide-binding (NB) domain and variable C-terminal leucine-rich repeats (LRR) domain [28]. In terms of their distinct N-terminal domains, NLRs are broadly divided into two groups: TNLs that harbour a Toll–interleukin 1 receptor (TIR) domain and CNLs that contain a coiled-coil (CC) domain [69–71]. NLRs belong to signal transduction ATPases with numerous domains (STAND) that operate as molecular switches cycling between an inactive closed ADP-bound state and active open state with bound GTP [72–74]. In the resting state, N-terminal TIR or CC and C-terminal LRR domains sterically inhibit the NB domain from ADP–ATP exchange. On pathogen recognition, a series of conformational changes occur that expose the NB domain, promote ADP–ATP exchange and initiate signal transduction [8]. Effector recognition by NLRs often, but not always, leads to a form of programmed cell death termed as a hypersensitive response [75]. NLRs are, therefore, under precise control by accessory proteins. NLRs interact with conserved Hsp90-Sgt1-RAR1 protein complexes for proper folding, accumulation and

regulation [76,77]. Moreover, Sgt1 interacts with the suppressor of *rps4-RLD* (SRFR1) negatively to regulate NLRs accumulation and prevent autoimmune activation [78].

Various strategies of effector recognition by NLRs exist and are represented in different models. In the gene-for-gene model, originally described by Flor in 1971, NLRs can recognize effectors directly (e.g. *Pita-AvrPita* [79], *Pto-AvrPto* [80] and many others) acting like receptor and ligand pairs that initiate a cascade of reactions leading to resistance [3]. When considering pathogen effector diversity, it is more likely that a single NLR recognizes multiple effectors from diverse pathogens in the presence of other host proteins. This hypothesis is explained by the guard model, in which the NLR protein is assigned the role of a sentinel that is activated indirectly by detecting an effector-modified host protein and induces a defence response [81,82]. An example of such mechanism is given by *Arabidopsis* CNL receptors, RPM1 and RPS2, which constantly monitor host protein RIN4 (a negative regulator of basal resistance) for interference with *Pseudomonas syringae* effectors AvrB, AvrRpm1 and AvrRpt2 [83]. Another indirect effector recognition strategy is proposed in the decoy model [84]. As guarded effector targets are evolutionarily unstable, it is likely that the targeted host gene has duplicated and evolved into decoy proteins. These serve as bait to trap effectors that target structurally related proteins involved in basal defence. For example, in the inactive state, *Arabidopsis* CNL RPS5 interacts with its N-terminal CC domain with protein kinase PBS1, which has no function in basal resistance [85]. Cleavage of PBS1 by *Ps. syringae* effector AvrPphB activates RPS5 [86]. However, several PBS1-like kinases (including Botrytis-induced kinase 1 (BIK1)) are also cleaved by AvrPphB [87]. PBS1, therefore, acts as a decoy that prevents cleavage of BIK1, which is an important component of PRR signalling [53] and the key AvrPphB target. An integrated decoy model has recently been proposed [5]. In this model, the effector-targeted plant protein is duplicated and fused to one member of the NLR pair to act as bait that, on effector binding, triggers defence signalling by the second NLR.

Activated NLRs trigger a variety of immune responses, from the generation of ROS, elevation of intracellular Ca^{2+}, activation of MAPK cascades, transcriptional reprogramming to production of phytohormones [8]. Although effector-triggered responses are qualitatively similar to immune responses elicited by MAMPs/DAMPs, there are quantitative differences in the strength and duration of pathways, which result in different resistance responses and signalling networks [17].

2.3. Signal transduction cascades

Perception of MAMPs/DAMPs by their cognate receptors triggers an array of immune responses, comprising changes in intracellular calcium levels $[Ca^{2+}]_i$, membrane potential depolarization, extracellular alkalinization, production of ROS, NO and phosphatidic acid, activation of kinases (mitogen-activated protein kinases (MAPKs) or Ca^{2+}-dependent protein kinases (CDPKs)), transcriptional reprogramming and changes in plant hormone concentrations (e.g. ethylene, salicylic and jasmonic acid) [88,89].

2.3.1. Calcium and ROS signalling interconnection

Ca^{2+} is a ubiquitous second messenger released in response to various stresses and developmental processes. In *Arabidopsis*, various MAMPs/DAMPs induce distinct and sustained

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elevations of intracellular calcium concentration ($[Ca^{2+}]_i$), which differ in the lag phase and amplitude of response [89,90]. Moreover, changes in $[Ca^{2+}]_i$ are organ-specific and correlate with the expression patterns of the corresponding MAMP/DAMP receptors. Chitin octamer and Pep1 induce similar responses in seedling shoots and roots, while roots are insensitive to elf18 and show only a minor response to flg22 [90]. Furthermore, Ca^{2+} fluxes are generated from different sources; flg22/FLS2 signalling involves the release of Ca^{2+} from intracellular stores (e.g. endoplasmic reticulum and/or tonoplast) and inositol phosphate signalling, whereas Pep/PEPR signalling requires an influx of Ca^{2+} from the apoplast [68]. The identity of plant Ca^{2+} channels and pumps involved in the generation of Ca^{2+} signals is largely unknown, although some candidates (e.g. ionotropic glutamate receptor (iGluR)-like channels, cyclic nucleotide gated channels (CNGCs) and annexins in plasma membrane and two-pore-channel 1 (TPC1) in the tonoplast membrane) have been investigated [88,91]. Elevated $[Ca^{2+}]_i$ is detected by Ca^{2+}sensor proteins such as calmodulins (CaMs), calcium-dependent protein kinases (CDPKs), calcineurin B-like (CBL) proteins and CBL-interacting protein kinases (CIPKs) [88,91]. CaMs are highly conserved eukaryotic proteins that bind free Ca^{2+} with four EF-hand motifs and regulate the function of their interacting proteins, such as CaM-binding transcription factors [88]. CDPKs are unique proteins acting as sensors and decoders of Ca^{2+} signals and are suited for rapid responses to stimuli. Binding of Ca^{2+} via four EF-hand domain motifs in the C-terminus activates the CDPKs' N-terminal kinase domain and promotes transmission of a Ca^{2+} signal by phosphorylating different target proteins [91]. The functional specificity of CDPKs is achieved by targeting distinct membrane subdomains and involves specific lipid modifications (e.g. N-terminal myristoylation, S-acylation) [91]. In contrast to CDPKs, CBLs are Ca^{2+} sensors without enzymatic activity. They bind Ca^{2+} with four EF hands and then associate with CIPKs through the NAF motif in the kinase C-terminal regulatory domain. This interaction liberates kinase from auto-inhibition and enables conversion of the Ca^{2+} signal into phosphorylation events [91]. Like CDPKs, CBLs have different lipid modifications (e.g. N-terminal myristoylation, S-acylation) that determine their localization and, consequently, the site of action of CBL–CIPK complexes [91].

Rapid production of reactive oxygen species (ROS) in response to MAMPs/DAMPs depends primarily on respiratory burst oxidase homologues (RBOHs) [92,93]. These NADPH oxidases are integral membrane proteins that generate superoxide anions (O_2^-), which are rapidly converted into hydrogen peroxide (H_2O_2). RBOHs have cytosolic FAD- and NADPH-binding domains in the C-terminal region, six membrane-spanning domains and a cytosolic N-terminal extension that harbours two EF-hand motifs and multiple phosphorylation sites [94]. Recent studies in *Arabidopsis* have revealed different regulation mechanisms of RBOHD and RBOHF-dependent ROS production. RBOHF regulation involves direct binding of Ca^{2+} to its EF-hands and Ca^{2+}-dependent phosphorylation by CBL1/9–CIPK26 complexes [95]. Direct binding of $Ca^{2+}$to EF-hand motifs on MAMP-induced elevation of $[Ca^{2+}]_i$ causes conformational changes and activation of RBOHD [96]. Additionally, RBOHD is activated by protein phosphorylation at multiple sites via calcium-dependent protein kinase 5 (CPK5)[97,98] and MAMP-receptor-associated Botrytis-induced kinase 1 (BIK1) [53]. In addition to local defences, Ca^{2+} and

RBOHD-dependent ROS production is implicated in the systemic signal propagation required for long-distance signalling [98–100]. In accordance with the current model [98], the perception of MAMPs triggers a rapid rise of $[Ca^{2+}]_{i,}$ causing the activation of CPK5 and subsequent phosphorylation of RBOHD and other CPK5 substrates. Apoplastic H_2O_2, generated after dismutation of the O_2^- produced by the RBOHD, probably represents the cell-permeable signal, which serves as the stimulus for further reiterations of calcium-dependent CPK5 activation and RBOHD phosphorylation, resulting in rapid propagation of the MAMP signal throughout the plant.

2.3.2. MAPK signalling

Plant mitogen-activated protein kinase (MAPK) cascades generally comprise MAPKK kinases (or MEKKs), which receive signals from receptors/sensors and phosphorylate downstream MAPK kinases (or MKKs) and which subsequently activate MAPKs (or MPKs) that control the activities and synthesis of a plethora of transcription factors (TFs), enzymes, hormones, peptides and antimicrobial chemicals [101,102]. In *Arabidopsis*, two kinase cascades, MKK4/MKK5–MPK3/MPK6 [103] and MEKK1–MKK1/MKK2–-MPK4, [104] are activated after perception of MAMPs/DAMPs. The activation of MEKK1–MKK1/MKK2–MPK4 negatively regulates ROS and salicylic acid (SA) production [105,106], as well as repressing cell death and immune responses [107]. MKK4/MKK5–MPK3/MPK6 cascade positively regulates the expression of several defence–related genes [60,103] and promotes accumulation of camalexin via transcription factor WRKY33 [108,109]. Moreover, activation of MPK3/MPK6 is required for full priming of stress responses [110] and increases ethylene production via ACC synthases ACS2/ACS6 [111]. Given the essential nature of the MKK4/MKK5–MPK3/MPK6 cascade, its activation has to be precisely controlled, since inappropriate activation (e.g. constitutively activated MKK4/MKK5 [112] or over-expression of MPK3 [113]) may promote hypersensitive response (HR)-like cell death or be lethal to plants. MPK3 has also recently been indicated to be a negative regulator of defence gene expression, flg22-triggered SA accumulation and disease resistance to *Pseudomonas syringae* [114]. Another negative regulator of MAPK activities is MAPK phosphatase 2 (MKP2), which interacts with and dephosphorylates MPK3 and MPK6 [115]. Additionally, a Raf-like MAPKK kinase (EDR1) has been proposed to negatively regulate the MKK4/MKK5–MPK3/MPK6 cascade by physically interacting with MKK4 and MKK5 via its N-terminal domain [113].

2.4. Transcriptional reprogramming converges with complex phytohormone signalling networks

Transcription factors (TFs) involved in plant immunity reside in transcriptional complexes and, together with co-regulatory proteins, directly or indirectly recruit RNA polymerase II to the target promoters or release it from them [116]. TFs vital for plant immunity comprise members of the AP2/ERF, bHLH, bZIP, MYB, NAC and WRKY TF families and perform diverse roles [9]. For instance, certain members of apetala2/ethylene-response element binding factor (AP2/ERF) participate in the regulation of genes related to the jasmonic acid (JA) and

ethylene hormone signalling pathways [117]. *At*MYC2/JAI1/JIN1 and closely related proteins *At*MYC3 and *At*MYC4 belong to basic-helix-loop-helix (bHLH) TFs and coordinate JA-mediated defence responses with other phytohormones (salicylic acid (SA), abscisic acid (ABA), gibberellins (GA) and auxin) [118]. TGA/basic domain leucine zipper (bZIP) family members are central players in SA-mediated resistance to biotrophic pathogens. Moreover, *At*TGA2, 5 and 6 TF have central roles in establishing systemic acquired resistance (SAR), regulate host detoxification pathways and are essential activators of certain ethylene-induced defence responses [119].

TF expression and activities are regulated in multiple ways. Certain nucleotide-binding site/leucine-rich repeat (NLR) proteins directly regulate transcription by physically interacting with TFs [120–123]. Several TFs are controlled by phosphorylation as downstream targets of activated MAPK cascades [109,124–127]. Another mechanism of TFs activation is carried out by Ca^{2+}sensors such as CaMs and CDPKs [128–133]. Additional factors (e.g. components of mediator complex [134,135], chromatin modifications [136–138]) and levels of regulation (e.g. ubiquitination [139], sumoylation [140], alternative mRNA splicing [141]) also contribute to the complexity of transcriptional networks and fine-tuning of immune responses.

Coordination of diverse stress responses and growth is resolved within complex phytohormone signalling networks, in which salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) perform central roles, while other hormones merely modulate their responses [142]. SA is synthesized in chloroplasts from chorismate by isochorismate synthase [143] and exported to the cytosol [144]. In response to pathogens and various abiotic stresses, SA mediates expression of pathogenesis-related (PR) genes and the synthesis of antimicrobial compounds to provide basal defence and systemic acquired resistance (SAR) [145]. SA activates these defence responses through transcription cofactor nonexpresser of PR genes 1 (NPR1) [146] and transcription factors TGA2, TGA5 and TGA6 [119]. In the absence of SA, NPR1 is sequestered in the cytosol and forms oligomeric complexes stabilized by intermolecular disulphide bonds [147]. In response to activation of SA pathway, thioredoxins reduce these disulphide bonds, causing the release of NPR1 in monomeric form, which can translocate to the nucleus via a nuclear translocation signal (NLS) [148]. In addition, NPR1 protein levels oscillate through $CUL3^{NPR3}$- and $CUL3^{NPR4}$-mediated degradation in the nucleus, which is required for fine-tuning of immune responses [147,149,150].

Jasmonates (JAs) are plant hormones with essential roles in plant defence and development [118]. JAs are derived from α-linolenic acid liberated from membrane phospholipids by the action of phospholipase A and enzymatically converted in a series of steps in chloroplasts and peroxisomes, to be finally transformed into bioactive molecule JA-isoleucine (JA-Ile) in the cytosol [151]. JA-signalling is activated after repressor removal [152,153]. In unstimulated cells, jasmonate ZIM domain (JAZ) proteins repress transcription of JA signalling components, such as the basic-helix–loop-helix (bHLH) master transcription factor MYC2 and its close homologues MYC3 and MYC4 [154]. On JA signal perception by coronatine insensitive 1 (COI1), a component of the Skp1-Cul-F-box protein (SCF) E3 ligase complex, JAZ repressor proteins are targeted for proteasome-mediated degradation and MYC2 activates the transcription of several JA-responsive genes [154,155].

Ethylene (ET) is a gaseous hormone that often works synergistically with JA [156]. Important steps in ET biosynthesis are the conversion of *S*-AdoMet to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase and oxidation of ACC by ACC oxidase to form ET [157]. In *Arabidopsis*, MPK3 and MPK6 phosphorylate ACS2 and ACS6 proteins to prevent rapid degradation of ACS2/ACS6 by the 26S proteasome pathway and enhance ET production in response to MAMP perception [111]. MPK3 and MPK6 also phosphorylate TF WRKY33, which subsequently binds to ACS promoters and regulates ET production [158]. Generated ET binds to its receptors, such as ethylene response 1 (ETR1) in the ER membrane. When ET is absent, active receptors ETR1 negatively regulate ethylene insensitive2 (EIN2) through phosphorylation via Raf-like protein kinase constitutive triple response1 (CTR1) [159]. At the same time, TFs ethylene insensitive3 (EIN3) and EIN3-like1 (EIL1) are recruited by two F-box proteins, EBF1 and EBF2, to 26S proteasomal degradation. On ET signal perception, the ETR1 receptors are inactivated and CTR1 repressed. Subsequently, the C-terminal part of EIN2 is cleaved and translocated to the nucleus [160]. This induces degradation of EBF1 and EBF2 and stabilizes EIN3 and EIL1, which regulate expression of ET-responsive genes (e.g. TF ERF1 and ORA59) [161,162].

Plant hormonal crosstalk is extensive and occurs in several combinations [163]. The molecular mechanism underlying SA-mediated reprogramming of the JA transcriptional network points to immune signalling antagonism and the involvement of transcriptional regulators NPR1, TGA, WRKY and ORA59 as signal integrators [164]. Phytohormones JA and ET synergistically regulate plant defence responses to necrotrophic fungi via JA-induced EIN3 and EIL1 activation and ET-induced EIN3 and EIL1 stabilization. In addition, antagonistic effects observed in JA and ET signalling are mediated by the interaction of JA-activated MYC2 TF and ET-stabilized TF EIN3 [165].

2.5. Inactivation of immune signalling pathways

Various negative regulation mechanisms ensure immune signalling activation is switched off when there is no danger. In the absence of ligand, several phosphatases interact with PRRs and their associated kinases to keep immune complexes inactive through dephosphorylation. For instance, Ser/Thr phosphatase type 2A (PP2A) constitutively associates with BRI1-associated kinase1 (BAK1) and negatively controls BAK1 activation in PRR immune receptor complexes [166]. Negative regulation exerted by downstream phosphatases is illustrated by *Arabidopsis* MAPK phosphatase 1 (MKP1) operating as a negative regulator of MPK6-mediated MAMP responses [167] and also observed with MAPK phosphatase 2 (MKP2), which acts as the key regulator of MPK3 and MPK6 networks controlling both abiotic and specific pathogen responses in plants [115].

Ubiquitination and proteasomal degradation are other mechanisms by which plant immune responses are attenuated. For example, two U-box E3 ubiquitin ligases, PUB12 and PUB13, are recruited to flagellin-induced FLS2/BAK1 receptor complex and phosphorylated by BAK1 to polyubiquitinate FLS2 and promote its degradation [168]. Downregulation of immune signalling can also be achieved by ligand-induced endocytosis and degradation [169]. This has

been reported in localization studies of flg22-induced FLS2 receptors in *Arabidopsis* [170] and was recently proposed as a mechanism for desensitizing host cells to flg22 stimulus and in turning over ligand-bound FLS2 [171].

3. Plant defence responses to xylem-invading pathogens

3.1. General perception of MAMPs in roots

Despite the fact that roots are subjected to a rich microbial community, the perception of MAMPs and immune responses in roots are poorly understood. Millet et al. [172] studied immune responses in *Arabidopsis* roots after exposure to flg22, PGN and chitin. Flg22 and PGN initiated signalling only in association with LRR RLK BAK1. Furthermore, bacterial flg22 and PGN triggered a localized response in the elongation zone of the root tip, while chitin induced a response only in the mature zones of roots. It is thus likely plants have evolved tissue-specific MAMP-triggered immune responses, depending on the nature of the attacker [172,173]. While fungi and nematodes can directly penetrate the epidermal layer of roots, bacteria cannot and therefore exploit the weakest part of the roots as infection site. This hypothesis has been confirmed by recent FLS2 expression studies [174], which indicated that basal FLS2 promotor activity is restricted to the vascular cylinder and outgrowing lateral roots. Moreover, the FLS2 receptor system in roots is functional, since flg22 treatment induced rapid calcium influx and caused phosphorylation of MAPK [174]. Whole transcriptome expression analysis of flg22-elicited roots also revealed a set of genes specifically upregulated in roots, with functions in hormone and stress signalling, root and lateral root development, signalling and defence [174].

3.2. Perception of vascular wilt pathogens

At early stages of infection, vascular wilt pathogens are faced with preformed physical and chemical root defences and MAMP-induced immune responses that hinder their invasion [20]. Once they breach the rigid secondary xylem walls and enter the xylem vessels, vascular wilt pathogens are presumably recognized by specific extracellular receptors in the parenchyma cells surrounding the xylem vessels [21].

3.2.1. Perception of Verticillium spp.

In tomato, extracellular LRR RLP Ve1 [24,175,176] plays a role in xylem defence and provides resistance against race 1 strains of *V. dahliae* and *V. albo-atrum* [177,178]. In recent years, several other homologue genes have been reported in *Gossypium*, *Solanum* and *Mentha*. A functional *Ve1* orthologue has also been discovered in *Nicotiana glutinosa* [179]. Ve1 recognizes a small effector protein, Ave1, with a similarity to plant natriuretic peptides involved in regulation of water and ion homeostasis [180]. Phylogenetic analysis has indicated hundreds of Ave1 homologues in plants but only a few in fungi, suggesting *Verticillium* spp. acquired Ave1 through horizontal gene transfer [180].

Ve1 forms heterodimers with a tomato orthologue of the *Arabidopsis* RLK suppressor of BIR1-1/evershed (SOBIR1/EVR) in the absence of Ave1 [181]. However, Ve1-mediated signalling also requires other critical signalling components, such as SERK1 and SERK3/BAK1, to establish *Verticillium* resistance in tomato and *Arabidopsis* [177,178,182]. Additionally, Ve1-mediated signalling depends on ER-QC-assisted folding mediated by ER-resident chaperones HSP70 binding proteins (BiPs) and lectin-type calreticulins (CRTs) [183].

3.2.2. Perception of Fusarium oxysporum f. sp. lycopersici

Three I (immunity) genes have been identified in tomato [184] in a resistance response to *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) that involves callose deposition, accumulation of phenolics and formation of tyloses and gels [185]. The *I-2* gene encodes an intracellular CC-NB-LRR receptor protein that perceives *Fol* effector protein Avr2 (secreted in xylem 3; Six3) [186]. Avr2 is under the control of transcription factor Sge1 and is highly expressed in roots and xylem vessels [187]. Avr2 forms homodimers and requires nuclear localization to trigger I-2-mediated cell death [188], which can be strongly suppressed by *Fol* effector Six6 [189]. It has recently been shown that Six5 also contributes to the virulence of *Fol* in tomato plants that Six5 and Avr2 can interact and are together required for *I-2*-mediated resistance [190].

3.2.3. Perception of Xanthomonas oryzae pv. oryzae

The rice LRR RLK Xa21 that provides resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) [191] recognizes sulphated peptide from the N-terminal part of the secreted quorum-sensing (QS) signal molecule activator of Xa21 (Ax21) [192,193]. In the absence of a signal, Xa21 associates with XB24, a protein with ATPase activity that enhances Xa21 autophosphorylation of Ser/Thr residues [194]. On Ax21 recognition, XB24 is released and Xa21 becomes activated to induce rice defence responses [193]. Subsequently, a protein phosphatase 2C (XB15) specifically interacts with activated Xa21, leading to dephosphorylation and inactivation of Xa21 [195]. In addition, several other proteins (e.g. RING finger ubiquitin ligase XB3 [196] and plant-specific ankyrin-repeat protein XB25 [197]) are associated with Xa21 and required for Xa21 accumulation and resistance to *Xoo* [193]. Moreover, Xa21 binds XB10, a WRKY62 transcription factor. When overexpressed, XB10 suppresses the activation of defence-related genes *OsPR1* and *OsPR10* and acts as a negative regulator of basal and Xa21-mediated immunity [198]. Xa21 also interacts with the endoplasmic reticulum (ER) chaperone BiP3, which regulates its stability and processing [199].

3.2.4. Perception of Ralstonia solanacearum

A pair of *Arabidopsis thaliana* TIR-NB-LRR proteins, RRS1 and RPS4, function together in disease resistance against *Colletotrichum higginsianum*, *Pseudomonas syringae* pv.*tomato* and *Ralstonia solanacearum* [200–202]. RRS1 and RPS4 proteins form an inactive heterodimer complex through the SH motif in their TIR domains [5,121]. RRS1 protein recognizes and, through its C-terminal WRKY domain, directly binds *R. solanacearum* effector PopP2 [203–205]. This leads to disruption of RRS1/RPS4 TIR heterodimer (but not full-length hetero-complex), allowing the formation of signalling active RPS4 TIR homodimer. PopP2 interacts

with other WRKY domain-containing proteins and acetylates lysines to block DNA binding, suggesting that PopP2 interferes with WRKY TF-dependent defence [205,206].

3.3. Induced defence responses to vascular wilt pathogens

Recognition of vascular wilt pathogens by plant immune receptor complexes activates defence responses in the xylem vessels. Physical defence responses that confine pathogens from further spread comprise the formation of tyloses, accumulation of pectin-rich gels and gums, vascular coating and callose and secondary cell wall deposition [20,21]. An interesting adaptation to vascular wilt infection is vein clearing, a tissue-specific developmental programme leading to the formation of new xylem elements [207]. Furthermore, significant metabolic changes have been reported in response to xylem infection and involve the induction of pathogenesis-related (PR) proteins, peroxidases, proteases as well as the production of antimicrobial secondary metabolites such as phytoalexins, sulphur-containing compounds and phenolic compounds [20,21].

Studies of defence signalling in response to root pathogens have so far mainly focused on the leaves and have provided evidence that defence mechanisms involve similar signalling pathways (Ca^{2+}-signalling, induction of ROS and MAPK cascades, modulation of phytohormone signalling) [20]. Moreover, plant microarray and RNASeq studies have revealed that the interaction between vascular wilt pathogens and host plants involves transcriptional reprogramming of hundreds of genes [208–211]. Interestingly, in an incompatible interaction, only modest changes in gene and protein expression have been reported [210,212–214] and most of the differentially expressed genes have been repressed in roots rather than in leaves [211]. Moreover, genes implicated in photorespiration, hypoxia, glycoxylate metabolism and auxin signalling show inverse regulation on infection with the foliar pathogen *Cladosporium fulvum* or root pathogen *Verticillium dahliae* [210].

Genome-wide analyses on transcriptional and proteomic levels, together with functional characterization of individual genes, have revealed a convergence of signalling pathways in response to individual pathogens, in mostly controlled conditions. In the field, plants are simultaneously challenged by multiple stress factors, both biotic and abiotic. Even though signalling components of plant regulatory networks are partly shared in both and point to general stress response mechanisms, there is evidence of specific responses to combined stresses that are controlled by different signalling pathways and such studies may provide additional candidates for crop protection breeding [14–16,18].

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