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An emerging connected view: Phytocytokines in regulating stomatal, apoplastic, and vascular immunity

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Foliar pathogens exploit natural openings, such as stomata and hydathodes, to invade plants, multiply in the apoplast, and potentially spread through the vasculature. To counteract these threats, plants dynamically regulate stomatal movement and apoplastic water potential, influencing hydathode guttation and water transport. This review highlights recent advances in understanding how phytocytokines, plant small peptides with immunomodulatory functions, regulate these processes to limit pathogen entry and proliferation. Additionally, we discuss the coordinated actions of stomatal movement, hydathode guttation, and the vascular system in restricting pathogen entry, multiplication, and dissemination. We also explore future perspectives and key questions arising from these findings, aiming to advance our knowledge of plant immunity and improve disease resistance strategies.

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Introduction

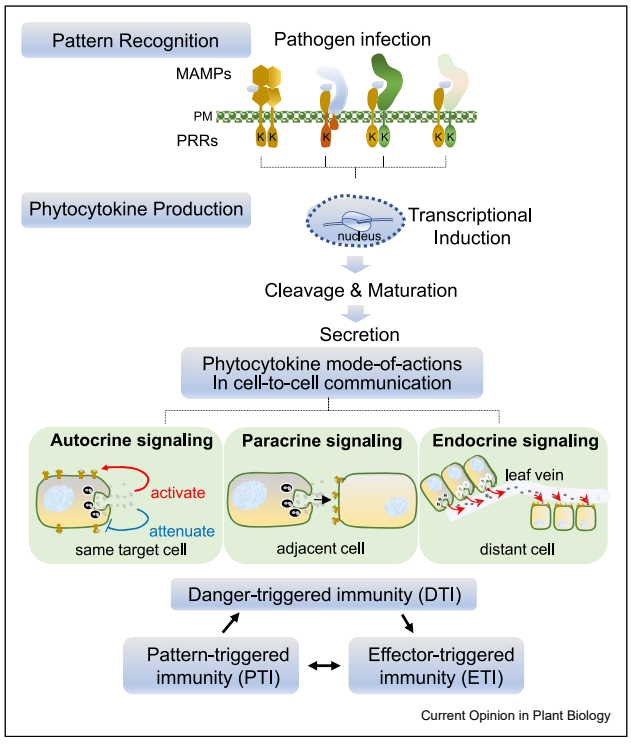
Pathogen infections or perception of microbe-associated molecular patterns (MAMPs) induce the production of immunomodulatory peptides, long known for their roles in plant growth, development and immunity, and later referred to as phytocytokines due to shared features with metazoan cytokines [1–4]. Phytocytokines can be induced at the transcriptional levels, undergo maturation by cleavage or posttranslational modifications, and then are released into the apoplast upon infections [4,5] (Figure 1). They serve as endogenous alarming signals, initiating danger-triggered immunity (DTI) [3,6,7]. Both MAMPs and phytocytokines are recognized by plasma membrane-resident pattern recognition receptors (PRRs), which are encoded by receptor-like proteins (RLPs) or kinases (RLKs) [8,9]. DTI signaling modulates MAMP-activated pattern-triggered immunity (PTI) and strengthens the intracellular nucleotide-binding domain leucine-rich repeat protein (NLR)-mediated effector-triggered immunity (ETI) [1,3,7].

Stomata, the natural openings on the leaf surface, facilitate gas and water exchange between plants and the atmosphere crucial for photosynthesis and transpiration. Stomata are exploited by leaf-invading microbes as entry points [10,11]. Consequently, plants and pathogens dynamically regulate the size of stomatal pores, enclosed by a pair of guard cells [11,12]. Upon sensing initial infection, plants respond to MAMPs and rapidly close stomata to restrict pathogen entry, known as stomatal immunity [10,13] (Figure 2a). In turn, pathogens have evolved counter-defense strategies, such as releasing toxins or effectors, to keep stomatal open, thereby facilitating pathogen entry [14].

Meantime, MAMP-induced stomatal closure creates a watery apoplast, which is conducive to bacterial multiplication [15,16]. Prolonged stomatal closure also decreases the transpiration rate, constraining plant productivity. To counteract these detrimental effects, plants induce specific phytocytokines to reopen stomata [17]. This mechanism, termed apoplastic immunity, helps to reduce water potentials, disrupting the pathogen-favorable aqueous environment for pathogen multiplication and lesion development [10,17] (Figure 2b).

Besides stomata, hydathodes, valves for the secretion of guttation drops at leaf margins and tips, represent large cavities for pathogen entry [18]. Hydathodes are connected to the vascular system through leaf veins, thus influencing the pathogen spread and apoplast water potential [19]. Unlike stomata, hydathodes cannot be fully closed but their aperture and exudation activities are regulated upon infections [20]. This regulation contributes to restricting pathogen

Figure 1



The production and mode-of-action of phytocytokines in plant immune responses. Pathogen infections or perception of microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) induce the transcription of phytocytokines, which undergo maturation by cleavage or posttranslational modifications and secrete into the apoplast. Upon recognition by PRRs, phytocytokines induce danger-triggered immunity (DTI). Phytocytokine-induced DTI signaling amplifies or modulates microbial pattern-triggered immunity (PTI), activates cell death in effector-triggered immunity (ETI), and potentially systemic acquired resistance (SAR). Phytocytokines, similar to cytokines in mammals, may act in autocrine (signaling to self), paracrine (signaling to neighboring cells), and endocrine (signaling to long-distance cells) manners to activate or attenuate immune responses.

dissemination to the vasculature, a phenomenon termed hydathode immunity [18,20] (Figure 2c).

Moreover, the vasculature, the primary transport system for water, nutrients, and minerals, also acts as a conduit for vascular pathogens [21–23]. The vascular system is connected with hydathodes, the water movement in the vasculature and hydathodes affects the water balance in the leaf apoplast, modulating apoplastic immunity [18,22]. Meantime, hydathodes and stomata drive the flow of xylem sap from roots to shoots, influencing the pathogen spread in the vasculature [19,24] (Figure 2d). Phytocytokines have been implicated in stomatal and apoplast immunity; however, their roles in hydathode and vascular immunity remain less understood. This review focuses on recent advances regarding the dynamic roles of phytocytokine-induced stomatal movements in preventing pathogen entry and restricting pathogen multiplication in the apoplast. We also explore how these mechanisms coordinate with hydathodes and the vascular system to regulate leaf water dynamics and limit pathogen spread and proliferation. Additionally, we discuss future perspectives and key questions arising from these findings.

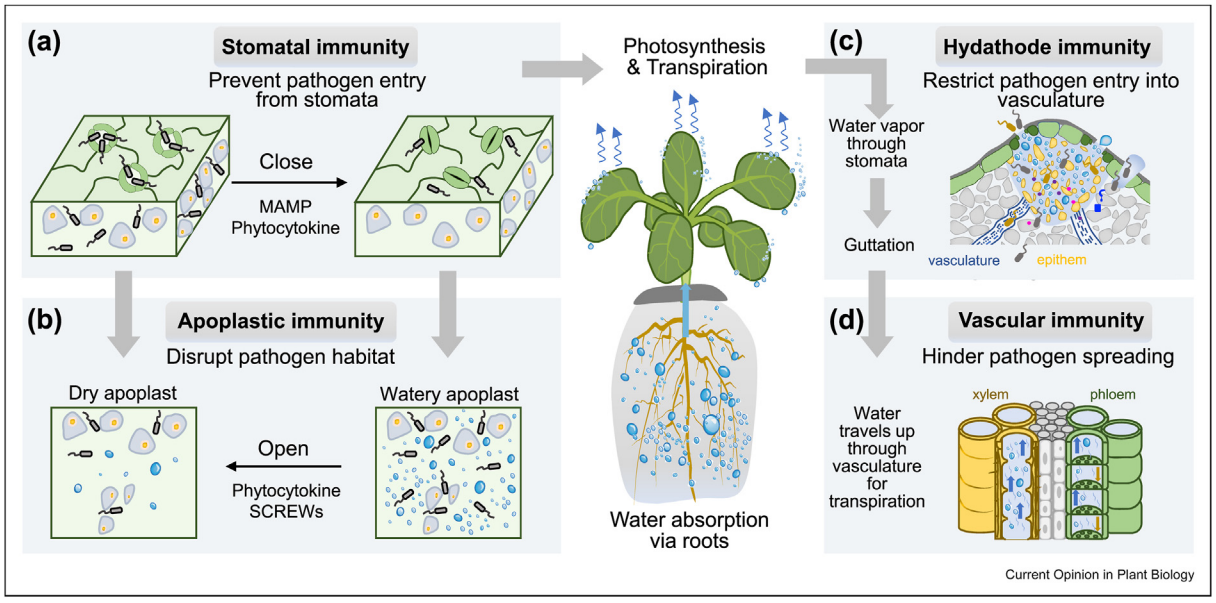
Phytocytokines trigger the stomatal closure

Many phytocytokines trigger largely overlapping immune responses with MAMPs, including rapid stomatal closure, to amplify PTI [7] (Figure 2a). However, the temporal and spatial dynamics of phytocytokine production in regulating stomatal movement during pathogen infections remain elusive. PLANT ELICITOR PEPTIDE1 (Pep1), a pioneer immune modulator in plant defense against herbivores and pathogens, is released from PRECURSOR OF PEP1 (PROPEP1) via the cleavage by Ca^{2+}-dependent metacaspases (MCs) upon wounding and microbial attacks [25,26]. Damage of *Arabidopsis* cells leads to a massive and prolonged influx of extracellular Ca^{2+} into the cytosol to activate MCs, which cleaves vacuolar membrane-bound PROPEP1 to release Pep1 into the cytosol [25], subsequently exported into the apoplast. Upon recognition by two closely related RLKs, Pep RECEPTOR 1 (PEPR1) and PEPR2, Pep1 triggers stomatal closure by enhancing the activities of guard cell-expressed S-type anion channels, including SLOW ANION CHANNEL 1 (SLAC1) and SLAC1 HOMOLOG 3 (SLAH3), yet independent of OPEN STOMATA1 (OST1), a key kinase mediating plant hormone abscisic acid (ABA)- and MAMP-induced stomatal closure [27]. In contrast, PAMP-INDUCED PEPTIDE 1 (PIP1), perceived by RLK7, initiates stomatal closure to defend against pathogen invasion by activating SLAC1 via the canonical OST1 pathway [28,29]. However, the mode-of-actions of most other phytocytokines in regulating stomatal closure remain to be explored.

Phytocytokines promote apoplastic immunity by reopening MAMP-induced stomatal closure

Stomatal closure during pathogen infections is a transient process, initially closing and subsequently reopening [10]. On the plant side, the reopening of stomata enhances water loss and dries the apoplast, thereby constraining pathogen proliferation [30] (Figure 2b). SMALL PHYTOCYTOKINES REGULATING DEFENSE AND WATER LOSSs (SCREWs), also called CTNIPs based on the conserved amino acid residues, are induced at the transcriptional level upon bacterial infections, insect infestations, and drought stresses [17,31,32]. Perceived by RLK PLANT SCREW UNRESPONSIVE RECEPTOR (NUT)/HAESA-LIKE 3 (HSL3), SCREWs counteract MAMP-induced stomatal closure by triggering NUT-dependent phosphorylation of ABA INSENSITIVE 1 (ABI1) and ABI2,

Figure 2



The coordinated action of stomatal, apoplastic, hydathode, and vascular immunity through phytocytokines. (**a**) During the initial infection stage, stomatal immunity restricts foliar pathogen entry into the apoplast through MAMP- and phytocytokine-induced stomatal closure. Phytocytokines, including Pep1 and PIPs, work alongside MAMPs to induce stomatal closure, thereby preventing subsequent pathogen entry.
(**b**) At the postinvasion stage, phytocytokines trigger stomatal reopening, thereby reducing apoplastic water potential and disrupting the watery habitat favorable to pathogens, contributing to apoplastic immunity. Phytocytokines, such as SCREWs, reopen closed stomata to promote water loss, thus limiting pathogen proliferation in the apoplast.
(**c**) Hydathode immunity limits pathogen entry through guttation and restricts subsequent spread via leaf veins for nonvasculature-adapted, but apoplast-adapted pathogens. However, this mechanism does not restrict vasculature-adapted pathogens. The hydathode structure is illustrated with large chambers in light blue beneath the pores, epithem in yellow, an epidermal layer in light green, mesophyll cells in grey, and vascular tissues in blue lines.
(**d**) Vascular immunity hinders pathogen spread within the vasculature. The vascular tissue consists of xylem, which transports water upward, and phloem, which transports metabolites bidirectionally. Stomata-mediated transpiration is the major driving force for water transport through the xylem, influencing hydathode guttation and the spread of vascular pathogens.

leading to an increased ABI phosphatase activity towards OST1 [17]. Analysis using an apoplastic water potential-responding reporter revealed elevated water potential levels in *nut* mutants compared to wild-type (WT) plants upon infection by bacterial *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000, corroborating the role of SCREW-activated signaling in modulating stomatal reopening and apoplastic water levels to enhance plant immunity [17]. Consistent with the drought-induced transcriptional upregulation, SCREWs also reopen stomata closed by ABA, a key hormone that induces stomatal closure to limit water loss upon drought stresses [17,31]. In addition, the rice ABA-deficient mutant *Osaba1*, which exhibits increased stomatal conductance, a parameter for stomatal opening, shows increased resistance to the bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) [33]. Although the involvement of phytocytokines in this process remains unknown, this study echoes the significance of stomatal opening status in enhancing apoplastic immunity against foliar pathogens during the postinvasion stage (Figure 2b).

Pathogens close stomata to disrupt apoplastic immunity for virulence

Water availability in the apoplast is crucial for pathogen colonization [24,34]. Pathogens have evolved strategies to establish aqueous apoplast and induce water-soaking symptoms via secreted effectors, such as avirulence E1 (AvrE1) and Hrp outer protein M1 (HopM1) [15]. These effectors induce stomatal closure by promoting ABA accumulation or manipulating the ABA signaling pathway, leading to water-soaking of leaves during infection [35–37]. Structural analysis indicated that AvrE-family effectors form water-permeable channels, altering osmotic/water potential and enriching the apoplast with water and nutrients, ultimately promoting bacterial multiplication within plant tissues [38]. Interestingly, some *Xanthomonas* transcription activator-like (TAL) effectors only affect water-soaked disease lesion development but not *in planta* bacterial multiplication [39,40]. These effectors may promote water uptake to enhance tissue damage and facilitate bacterial egression from the apoplast to the leaf surface [40]. These studies highlight the apoplast as a crucial

battleground for plant immunity and pathogen virulence.

Hydathode immunity protects the leaf vasculature against bacterial pathogen colonization

Hydathodes excrete xylem guttation saps from large pores at their surface when root pressure exceeds the leaf transpiration rate. The re-uptake of this guttation fluid provides an entry point for pathogens into extracellular cavity [18,20] (Figure 2c). Gummy guttation fluid plays a role in limiting pathogen entry via hydathodes [18]. Unlike stomata, hydathodes do not fully close in response to bacterial MAMP flg22, questioning whether the hydathode pore plays an active role in limiting microbial entry into the cavities [20]. However, resembling stomata, hydathode apertures are responsive to ABA and light, and limit the proliferation of a disarmed *Xanthomonas campestris* pv. *campestris* (*Xcc*) *hrpG* mutant strain (type III secretion system-deficient) [20,41], implying a postinvasion immune response in hydathodes. Furthermore, some well-studied PRRs, such as FLAGELLIN SENSING 2 (FLS2) and LysM RECEPTOR-LIKE KINASE 4 (LYK4), perceiving MAMPs from bacterial flagellin and fungal chitin, respectively, are highly expressed in hydathodes [42]. Moreover, genes encoding phytocytokine PIP1 and the cognate RLK7 receptor are also highly expressed in hydathodes [29,43]. A recent report used spraying assays with bioluminescence-tagged, vascular-adapted bacterial *Xcc* and vascular-non-adapted but apoplast-adapted *Pst* strains to visualize bacterial dissemination in hydathodes and leaves. This study showed that while both *Xcc* and *Pst* colonized hydathodes via guttation, only *Xcc*, but not *Pst*, could escape from hydathodes toward the leaf vasculature [44]. By screening various *Arabidopsis* immune-compromised mutants, the authors revealed that immune signaling nodes, including RLKs BRASSINOSTEROID-INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1) and SUPPRESSOR OF BIR1 (SORBIR1), shared co-receptors for multiple PRRs, and the ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1)-PHYTOALEXIN-DEFICIENT 4 (PAD4)-ACTIVATED DISEASE RESISTANCE 1 (ADR1) module, activated by multiple NLRs, are crucial for restricting both the initial hydathode colonization and subsequent spread of *Xcc* via the leaf vasculature [44]. This suggests the involvement of both plant PTI and ETI in this process. The connection between PTI and ETI through phytocytokine-mediated DTI [7] suggests that phytocytokines might be involved in regulating hydathode immunity as a post-invasion defense mechanism, a largely unexplored area.

Vascular immunity interplays with stomatal, apoplastic, and hydathode defense

Stomatal movement and hydathode guttation influence the transpiration rate, affecting the water potential in the vascular system, where vascular pathogens reside [18,24] (Figure 2d). Concurrently, as the xylem mediates water transport throughout plants, the collective action of stomata, hydathodes, and the vasculature system regulates the water potential in the apoplast, affecting foliar pathogen entry, multiplication and contributing to apoplastic immunity [18,19,24] (Figure 2). Notable vascular defense strategies include inducible physico-chemical barriers, such as the deposition of lignin or the formation of tyloses in vessels, to restrict pathogen spread [45,46]. Additionally, the vascular system serves as a conduit for immune signals, such as methyl-salicylic acid (SA), which are transported from the site of infection to systemic, uninfected tissues [47–50]. While it remains unclear whether phytocytokines can be transported through the vascular system as mobile signals, some phytocytokines, such as PEPs and SYSTEMINs, have been shown to trigger immune responses in distant tissues [51,52].

Looking ahead: what we know and what's next

1. What determines the functional specificity and redundancy of phytocytokine cocktails?

Plant genomes contain hundreds to thousands of genes encoding small secreted peptides, yet the functions of most of these peptides remain unclear, with only a small subset known to be involved in plant immunity [2,53]. It is crucial to systemically identify the *bona fide* phytocytokines secreted into the apoplast, hydathodes and vasculature during different stresses. Phytocytokines often exist as multimember protein families with loosely conserved functional motifs. For instance, the SCREW family contains four members, and SERINE RICH ENDOGENOUS PEPTIDE (SCOOP) family has at least 50 members in *Arabidopsis thaliana* [17,54]. It is unclear whether and how they function additively, synergistically, or antagonistically.

Unlike peptides involved in plant growth and development, the tissue- and cell-type specificity of phytocytokines is poorly characterized, particularly in the context of stomata, hydathode, and vascular systems. It remains to be explored whether phytocytokines are expressed and function differently in infected cells compared to naïve cells without direct contact with pathogens. For instance, do surface epidermis cells respond to MAMPs and phytocytokines similarly to inner endodermis cell layers? In addition, plant roots are classified by different developmental zones with distinct features. Do different zones exhibit similar responses to various phytocytokines?

Single-cell-based omics can identify cell-type-specific cellular responses in plant-microbe interactions

[55,56]. Spatial transcriptomic analysis, combined with cell-type-specific gene editing of multiple family members and innovative infection assays, will aid in the systemic characterization of the involvement of phytocytokines in stomatal, hydathode, and vascular immunity. Furthermore, employing sensitive peptide detection methods, such as ribosome footprinting capable of capturing peptides from short upstream open reading frames (uORFs) [57,58], could enhance the profiling of novel small peptides found in vascular or hydathode fluids during pathogen infections.

2. What are the mode-of-actions of phytocytokines in cell-to-cell communications?

Unlike cytokine-mediated cellular communication in mammals, which includes autocrine (signaling to self), paracrine (signaling to nearby cells), and endocrine (signaling to distant cells) pathways, the ways in which phytocytokines communicate among plant cells still remain largely uncharacterized (Figure 1).

The evidence supporting the long-distance transport of phytocytokines is currently limited. For instance, biotin-labeled SCREW1 peptides, despite being expressed in vascular tissues, were not detected in distal leaves and did not induce immune responses in systemic leaves [17], suggesting their autocrine or paracrine functions. Similarly, although PEP1 triggers systemic immune responses, its direct transport was not detected [51]. This suggests that phytocytokines may not move themselves over long distances but instead elicit signaling events in nearby cells, leading to the production of secondary messengers and metabolites that act as mobile signals, ultimately triggering immune responses in distant tissues.

Rapid calcium spikes have been proposed as a mobile signal in the wounding response [59] and defense hormone methyl-SA can be transported to systemic tissues [47,48]. Notably, CLAVATA3/ENDOSPERM SURROUNDING REGION-RELATED 25 (CLE25) moves from roots to shoots, serving as long-distance signals in mediating ABA-induced stomatal closure during dehydration stress [60]. It is conceivable that only minute quantities of individual phytocytokines are transported to distant tissues, potentially too faint to be detected with current techniques. Enhancing detection sensitivity and resolution at the single-cell level could offer insights into the mechanisms through which phytocytokines operate in cell-to-cell communication.

3. How do phytocytokines mediate the cross-talk between biotic and abiotic stresses?

Plants confront various environmental challenges concurrently, and stomata serve as crucial communication portals between plants and their surroundings. Despite our understanding of the molecular mechanisms governing stomatal movement under individual stress conditions, the coordinated impact of simultaneous abiotic stresses, particularly dehydration, and biotic stresses on stomatal movement, potentially regulated by phytocytokines, remains largely unexplored. For instance, the SCREW-NUT ligand-receptor pair positively regulates plant immunity but negatively impacts dehydration tolerance by counteracting both ABA- and MAMP-induced stomatal closure [17,31]. Structure modeling and molecular simulation, coupled with mutational and functional analysis, may aid in mechanistically understanding how SCREW-NUT pairs regulate immunity and drought stress.

Given that drought-induced, root-derived CLE25 peptides are transported into shoots to regulate stomatal closure in response to ABA [60], it would be interesting to investigate whether CLE25 or other peptides are induced by soil-borne root pathogens and regulate stomatal or vascular immunity to foliar pathogens in leaves, potentially contributing to systemic acquired resistance against difference types of pathogens. Recent research has shown that upregulation of the immune responsive genes upon drought recovery, termed drought recovery-induced immunity (DRII), confers resistance to rehydrated plants [61]. The involvement of phytocytokines in the DRII process remains unknown.

In addition, PEPs are well-known for their roles in plant immunity against bacteria, fungi, and herbivores [62]. Although the underlying mechanism is not clear, PEP3 also positively regulates responses to salinity stress [63]. Therefore, it would be reasonable to engineer PEP-PEPR ligand-receptor pairs to increase resistance to pathogens, insects, and salt stress.

4. How do phytocytokines coordinate stomatal, apoplastic, hydathode, and vascular immunity?

Stomatal movement is connected to hydathodes and the vascular system through apoplast water potential changes. The SCREW-NUT system not only regulates plant immunity to foliar pathogens by reducing apoplastic water levels but also contributes to resistance against phloem-feeding, sap-sucking insects, such as the green peach aphid (*Myzus persicae*) [17]. These aphids infest plants by using specialized mouthparts to penetrate plant tissues and extract sap from the phloem, creating negative pressure through a pump in their head as a passive feeding behavior [64,65]. Therefore, water potentials in the plant vascular system influence the infestation of sap-sucking insects.

SCREWs are induced upon aphid feeding and are expressed in vascular tissues [17], suggesting a potential role in coordinating apoplastic and vascular immunity by regulating stomatal movement and changes in apoplast water potential. Changes in water potential in the vascular system and guttation in hydathodes may increase the mobility of phytocytokines. It is also plausible that phytocytokine genes could be transcriptionally regulated in response to water potential changes, thereby mounting integrated stomatal, apoplastic, hydathode, and vascular immune responses.

5. Is phytocytokine-mediated stomatal immunity involved in ETI?

Stomatal immunity is well recognized in plant PTI. Whether and how it is involved in ETI remains an open question. A recent study shows that the EDS1/PAD4-ADR1s module, a key component activated by NLRs, regulates pathogen- and flg22-induced stomatal closure and confers resistance to virulent bacterial *Pst* spray infections. Additionally, *Pst* infections induce ADR1 interaction with EDS1/PAD4 in *Nicotiana benthamiana* guard cells [66]. Since the EDS1/PAD4-ADR1s module connects PTI and ETI [67,68], it would be interesting to know whether EDS1/PAD4-ADR1s-mediated stomatal immunity is a result of PTI or if it is also involved in ETI activated by sensor NLRs. The phytocytokine-mediated DTI amplifies PTI, leading to EDS1/PAD4-ADR1s-dependent ETI [67], suggesting the potential involvement of phytocytokines in EDS1/PAD4-ADR1s-mediated stomatal immunity. Furthermore, the effector AvrRpt2-triggered ETI blocks *Pst*-induced apoplastic water soaking to promote immunity [15]. It will be intriguing to investigate whether AvrRpt2 regulates water soaking by manipulating stomatal movement.

In contrast to fungal chitin-induced stomatal closure, chitosan, converted from chitin oligosaccharides by chitin deacetylases from fungi, does not induce stomatal closure [69]. Instead, an elevated concentration of chitosan induces cell death in guard cells, thereby impeding fungal invasion through stomata [69]. Interestingly, chitosan-triggered guard cell death does not depend on the chitin receptor CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) [69]. Localized cell death is a hallmark of plant ETI. It would be worthwhile to determine whether chitosan-triggered cell death is mediated by NLRs, or if chitosan induces massive phytocytokine production in guard cells, leading to cell death.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Data availability

No data were used for the research described in the article.

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- •• of outstanding interest

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