

## Spotlight

## A Conserved Microbial Motif 'Traps' Protease Activation in Host Immunity

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**A recent study (Misas-Villamil *et al.*, *Nat. Commun.*, 2019) reveals that Pit2, an apoplastic effector of the corn smut fungus *Ustilago maydis*, contains an embedded motif of 14 amino acids that binds to and inhibits plant cysteine proteases, thereby modulating host immunity. Intriguingly, the inhibitory motif acts by mimicking the protease substrate and is conserved across microbial kingdoms.**

### Papain-like Cysteine Proteases as Junctions in Immune Networks

Papain-like cysteine proteases (PLCPs) modulate diverse processes, including different layers of immunity, in both animals and plants. Animal PLCPs, also known as cathepsins, act either extracellularly or reside in endolysosomal systems under low-pH conditions, playing a vital role in cellular turnover [1]. PLCPs contain an autoinhibitory pro-domain and a protease domain, and are often secreted into the extracellular space by means of an N-terminal signal peptide. Upon maturation, the protease domain is cleaved to release the mature protease with the catalytic triad Cys-His-Asn. Plants have evolved nine PLCP subfamilies which function in the biotic stress response in pathogen-challenged host cells [2].

Previous studies revealed that PLCPs play a role in plant defense against several unrelated pathogens. Papain, a classical PLCP derived from the latex protease component

of papaya, has been associated with resistance to herbivore attack [3]. Knock-out of PLCPs suppresses plant immunity, rendering the host more susceptible to pathogenic invaders. For example, antisense lines of tomato for the PLCP Pip1 are more susceptible to taxonomically unrelated leaf pathogens such as *Cladosporium fulvum*, *Pseudomonas syringae*, and *Phytophthora infestans* [4]. The cellular pathways for PLCP-mediated activation of innate immunity are broadly conserved. Thus, the proteolytic activity of papain not only activates plant mitogen-activated protein kinase (MAPK) signaling but also activates protease receptors in mammalian cells including humans [5].

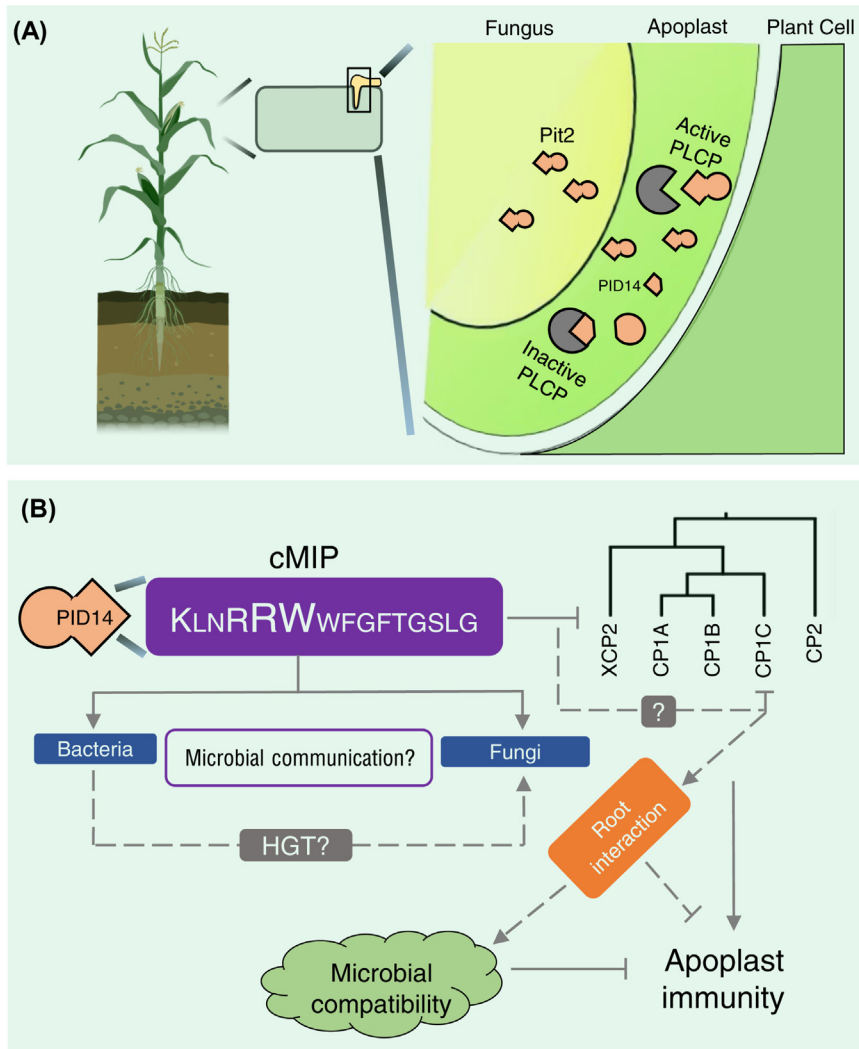
Compared with the aerial parts of the plant, much less is known about PLCP regulation in roots and their role in immunity. A recent study showed that the apoplast proteomes of leaf and root differ in PLCP activity [6]. Moreover, novel root-specific PLCPs, such as CP1C, have been associated with salicylic acid (SA) signaling in the root apoplast, and several PLCPs showed increased differential expression in roots compared with leaves, with CP1C showing the strongest upregulation [6]. Interestingly, CP1C is closely related to the PLCP Mir1 from maize, whose accumulation enhances resistance against root-feeding herbivores [7]. Collectively, these studies suggest an organ-specific role of PLCPs in immune regulation. How different PLCPs function in different root cell types, and how their activity triggers defense signaling, is an intriguing question. It is also unclear how the target specificity of PLCPs discriminates 'self' from 'non-self' proteins during the activation of cell death signaling.

### PLCPs Are Hijacked by Pathogen Effectors

The importance of PLCPs as components of host immunity is highlighted by the fact that their activity or subcellular localization

is targeted by diverse effectors from evolutionarily unrelated pathogens including fungi, oomycetes, bacteria, nematodes, and viruses. For example, *P. infestans*, a devastating oomycete pathogen which caused the great Irish famine, targets the potato protease C14 through the cystatin-like effectors EpiC1 and EpiC2B [8]. Another effector from the same pathogen, AvrBlb2, targets C14 protease of tomato, likely by blocking its secretion. A classic example of a PLCP targeted by diverse pathogen effectors is Rcr3 from tomato. This protein acts as a co-receptor in pathogen recognition and is suppressed by the previously described effectors EpiC1 and EpiC2B from *P. infestans*, as well as by the effector Avr2 from the extracellular pathogen *C. fulvum* and by the nematode *Globodera rostochiensis* [8]. In a further example, *Arabidopsis thaliana* PLCP RD19 is targeted by the bacterial type III effector PopP2 from *Ralstonia solanacearum* which interferes with its subcellular localization [8]. The apoplastic effector Pit2 from the biotrophic model fungus *U. maydis* was previously shown to suppress the activity of a set of maize proteases, including CP1A, CP1B, XCP2, and CP2 [9]. Intriguingly, these different PLCPs show hallmarks of natural variation in surface residues, which are located in close proximity to the active site, indicating an ongoing arms race for host adaptation to invading pathogens [10].

In the face of this strong selection pressure, pathogens have developed a striking strategy to counter PLCP variation and maintain successful infection. In the highlighted research, Misas-Villamil *et al.* show that the *U. maydis* effector Pit2 inhibits PLCP activity through a conserved amino acid motif, termed PID14, which is embedded in the Pit2 sequence and released by the action of maize PLCPs [11] (see Figure 1). Intriguingly, this motif binds to maize apoplast PLCPs with much higher affinity than the full-length Pit2 protein, suggesting that it acts as a



**Figure 1. Mechanism of Action of the Pit2 Effector in Suppressing Apoplast Immunity.** (A) The secreted effector Pit2 from *Ustilago maydis* mimics a cysteine protease substrate. Cleavage of Pit2 by proteases releases a 14 amino acid embedded motif (PID14) which efficiently binds to the active site of papain-like cysteine proteases (PLCPs) that function in activation of immunity. (B) Illustration of the 14 amino acid domain of Pit2 involved in suppressing maize apoplast proteases. This microbial inhibitor of proteases (cMIP) is conserved across the kingdoms of bacteria and fungi. Although the evolutionary origin of the cMIP motif is unknown, its presence in a pro-peptidase in *Burkholderia vietnamiensis* indicates that it may have emerged as part of a bacterial protease pro-peptide and was transferred to fungal pathogens by horizontal gene transfer (HGT). The cMIP motif might also be involved in microbial communication by modulating the activity of different apoplast proteases, including PLCPs such as CP1C which are specific to roots. Hence, cMIP represents a sophisticated strategy to suppress apoplast plant immunity for microbial compatibility. Part of this figure was created using BioRender ([biorender.com](https://www.biorender.com)).

molecular mimicry substrate that ‘traps’ plant PLCPs. This idea is supported by evidence from Pit2 modeling, which indicates that most PID14-domain amino acid residues including K44 and R47 are surface-exposed, thereby forming

a hook-like structure for efficient binding to the active target proteases. Upon cleavage, the substrate trap releases the PID14 motif, which binds to the PLCP substrate-binding pocket to block PLCP-mediated immune signaling.

Most strikingly, although the reported inhibitory function of Pit2 has so far been demonstrated only for the host plant maize, the PID14 motif was found throughout several taxonomically unrelated fungi and bacteria. The latter finding led the authors to propose that PID14 represents a new type of conserved microbial inhibitor of proteases (cMIP) that functions across host kingdoms to neutralize PLCPs and suppress immunity [11].

### Perspectives

PLCPs, in addition to other apoplast proteases, play crucial regulatory roles in host defense responses to biotic stresses, thereby determining the fate of the invading pathogen. Pathogens face strong selective pressure to neutralize this mechanism so as to successfully infect the plant host. The work by Misas-Villamil *et al.* demonstrates that cMIP is present not only in pathogens but also in endophytes. Therefore, inhibition of PLCPs by a conserved peptide represents a universal microbial strategy to suppress basal host defense responses and establish a compatible interaction. Because our knowledge of the role of PLCPs during root infection remains limited, this work opens up new avenues to explore how microbial effectors from root-infecting microbes, such as the vascular wilt pathogen *Fusarium oxysporum*, target apoplast proteases to suppress the root defense response. For example, the recent finding that *F. oxysporum* secretes plant regulatory peptides to increase host pH during infection [12] could be of relevance given that pH is known to play a crucial role in determining the proteolytic state of the cell. Moreover, because secreted proteases from several plant pathogens have been linked to infection, it will be interesting to explore whether any of these proteases integrate PID14-like domains for modulation of enzymatic activity during development.

In summary, the findings of Misas-Villamil and colleagues reveal a previously unrecognized microbial tool for suppressing plant defense in both mutualistic and pathogenic interactions. A key question concerns how the cMIP motif has evolved and is maintained in both endophytes and pathogens. The finding that the cMIP motif in *Burkholderia vietnamiensis* is annotated as part of a creatinase N-terminal domain suggests that its original role may have been to maintain Xaa-Pro-peptidases in an inactive state [11]. It is currently unclear whether the cMIP motif has been horizontally transferred from bacteria to fungal pathogens, or whether it evolved independently multiple times. Because plants in natural habitats are colonized by diverse microbes, an intriguing idea is that secreted cMIP protease inhibitors might function as a collective microbial strategy for communication and host manipulation in the apoplast. The identification of this novel motif hence provides a useful tool to study the substrate specificity of proteases across different plant organs, which is largely unknown.

Finally, because PLCPs display stunning variation as a result of the arms race against pathogens, an intriguing question

is how a single cMIP motif can cope with this array of natural variation in PLCPs. In this context, it would be interesting to study whether similar substrate-mimicry motifs have evolved to target different families of proteases that act in host immunity. Moreover, this evolved motif might presumably also be involved in amplifying the trigger for production of host protease inhibitors such as CC9 that inhibits cysteine proteases during infection [8]. These microbial probes will be helpful in understanding the regulation of proteases, and thereby provide a deeper insight into downstream signaling events that lead to the hypersensitive response (HR). By revealing a novel microbial strategy to establish host compatibility, the highlighted work expands our understanding of the link between plant proteases and microbial effectors, ultimately contributing to the success of these microbes in colonizing different hosts.

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