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Viewpoint

Hacking the immune system: plant immune protease engineering for crop protection

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Genome engineering technologies allow the generation of crops with increased disease resistance, though selecting suitable targets remains challenging. Our team has published two recent studies that highlight the potential of engineering plant immune proteases as an alternative approach to generating disease resistant plants.

Plant diseases threaten both cultivated and wild plants, jeopardizing food security and biodiversity (Singh *et al.*, 2023). Plants detect invading pathogens through receptors either at the plasma membrane (pattern-recognition receptors, PRRs) or inside the cell (NOD-like receptors, NLRs). Pathogen perception triggers robust immune responses, conferring resistance to most pathogens. Adapted pathogens use specialized molecules called effectors to cause disease by overcoming recognition and hampering defence responses among others. The genetic basis of disease resistance has been investigated for over a century uncovering various components of the plant immune system. Advances in genetic engineering are now enabling the application of this knowledge, expanding the genetic tools available to breeders. For example, transferring immune receptor genes between species has broadened pathogen recognition (van Esse *et al.*, 2020). This is possible because immune receptors have varied conservation patterns, resulting in some plants being able to 'see' certain pathogens whereas others are 'blind' to them (Ngou *et al.*, 2022), while the downstream signal components seem to be generally conserved.

Recent breakthroughs in structural biology such as AlphaFold (Jumper *et al.*, 2021), have further advanced our understanding of plant immunity, paving the way for precise modifications of immune proteins to enhance crop protection (Outram *et al.*, 2022). Efforts to engineer immune receptors continue (Marchal *et al.*, 2022b; Snoeck *et al.*, 2024), but two new studies demonstrate the potential of immune protease engineering to boost plant resistance through alternative mechanisms. Here we discuss the diverse roles that proteases have in immunity and discuss two proofs-of-concept on the engineering of immune protease *Phytophthora*-inhibited protease 1 (Pip1) as new directions for protease engineering.

Proteases have diverse roles in immunity and are targeted by pathogen effectors

Proteases, which hydrolyse peptide bonds, play essential roles in plant physiology, including immunity (Liu *et al.*, 2024). Plant immune proteases play crucial roles in several stages of plant defence, helping to protect plants against a wide range of pathogens. Proteases are found in various cellular compartments, including the apoplast, and are highly diverse, including serine, cysteine, aspartic, and metalloprotease classes (Balakireva and Zamyatnin, 2018). In the context of immunity, plant proteases have, in addition to endogenous substrates, also pathogen-produced substrates and can therefore have a direct impact on the invader (Wang *et al.*, 2019).

The diverse roles of proteases in immunity can be grouped into at least four categories: (i) pathogen perception, (ii) regulation of the immune response, (iii) counteracting pathogen effectors, and (iv) direct pathogen attack (Fig. 1). Plants perceive pathogens through the detection of the presence or activity of pathogen-derived molecules by immune receptors. Many of these molecules are distinct peptides derived from

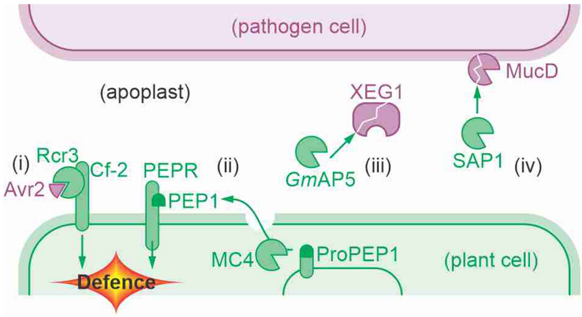

Fig. 1. Four classes of roles of immune proteases illustrated via examples. (i) Pathogen perception: Required for *Cladosporium* Resistance-3 (Rcr3) protease is inhibited by the fungal avirulence effector Avr2. The Rcr3-Avr2 complex is recognized by the immune receptor Cf-2, triggering a defence response (Kruger *et al*, 2002). (ii) Regulation of the immune response: METACASPASE 4 (MC4) is activated by calcium upon wounding or pathogen attack. MC4 cleaves tonoplast-located ProPEP1 releasing PEP1 to the apoplast where it is perceived by PEP RECEPTORS (PEPRs) thereby initiating defence responses (Hander *et al*, 2019). (iii) Counteracting pathogen effectors: soybean aspartic protease GmAP5, degrades the *Phytophthora sojae* virulence factor glycoside hydrolase family 12 (GH12) protein, XEG1 (Xia *et al.*, 2020). (iv) Direct pathogen attack: secreted aspartic proteases (SAPs) cleave *Pseudomonas syringae* MucD protein thereby suppressing bacterial growth (Wang *et al.*, 2019).

pathogen proteins and in at least two cases it has been shown that immune proteases are required for the generation of such immunopeptides (Wang *et al.*, 2021; Buscaill *et al.*, 2024). Hence, some immune proteases generate the immunopeptides required for pathogen perception thereby acting upstream of immune receptors and enabling pathogen perception in the first place.

Proteases can also serve as co-immune receptors as is the case for the extracellular tomato cysteine protease Rcr3 (Required for *Cladosporium* Resistance-3). Rcr3 is inhibited by the avirulence effector Avr2 which is secreted by the fungal pathogen *Cladosporium fulvum*. The Rcr3-Avr2 complex is recognized by the immune receptor Cf-2 on the cell surface, thereby triggering the hypersensitive response, a form of regulated cell death (Kruger *et al.*, 2002). Similarly, some integrated domains in NLRs (Marchal *et al.*, 2022a) constitute active proteases (Pedley *et al.*, 2019) although the role of the protease domain in pathogen perception remains to be elucidated.

Many immune proteases described to date act on the regulation of the immune response. Prominent examples include proteases enabling and regulating the HR (Salguero-Linares and Coll, 2019). Other regulatory immune proteases function in the generation of phytocytokines which are endogenous peptides acting in immunity (Gust *et al.*, 2017; Hander *et al.*, 2019).

Some immune proteases evolved to counteract pathogen-produced effectors and thereby attempt to disarm the pathogen and hold back the disease process (Reavy *et al.*, 2007; Xia *et al.*, 2020; Wang *et al.*, 2021). Soybean aspartic protease GmAP5, for instance, degrades endoglucanase XEG1 secreted by *Phytophthora sojae* (Xia *et al.*, 2020), whereas tomato subtilases like P69B cleave PC2, a small, Cys-rich effector secreted by *Phytophthora infestans* (Wang *et al.*, 2021).

Finally, some immune proteases directly disrupt pathogen growth and proliferation by targeting pathogen substrates (Fescemyer *et al.*, 2013; Wang *et al.*, 2019). Secreted aspartic proteases (SAPs) from Arabidopsis, for instance, cleave the MucD protein of the bacterial pathogen *Pseudomonas syringae* thereby suppressing bacterial growth (Wang *et al.*, 2019).

Given their critical roles in immunity, pathogens often evolve strategies to counteract immune proteases, such as producing inhibitors or degrading proteases. The tomato immune protease Pip1 was identified because it was inhibited by the effector EpiC2B of the blight pathogen *P. infestans* (Tian *et al.*, 2006). This papain-like cysteine protease (PLCP) is also inhibited by effectors from the pathogenic fungus *C. fulvum* and the bacterial pathogen *P. syringae* highlighting its role in immunity (Shabab *et al.*, 2008; Shindo *et al.*, 2016). Indeed, *Pip1* silencing increased susceptibility to *P. infestans*, *C. fulvum* and *P. syringae*, demonstrating that Pip1 is a general immune protease (Ilyas *et al.*, 2015). Recent studies have engineered Pip1 in two directions: to resist effector inhibition (Schuster *et al.*, 2024) and to act as a co-receptor for pathogen detection (Kourelis *et al.*, 2024).

Engineering inhibitor-insensitive immune proteases: ePip1

Proteases play crucial roles in plant immunity, but many pathogens secrete effectors to inhibit them. Engineering proteases to avoid inhibition by effectors without affecting protease function itself should hence enhance plant immunity, irrespective of the exact immune function and without compromising plant growth or development. We demonstrated this by engineering a variant of Pip1 that is resistant to inhibition by the cystatin-like EpiC2B effector, secreted by the late blight pathogen *P. infestans* (Fig. 2A). Expressing this engineered protease in *Nicotiana benthamiana* increased resistance to *P. infestans* (Schuster *et al.*, 2024). Notably, this approach does not require knowledge of substrates or molecular mechanisms, making it broadly applicable to other proteases involved in immunity. This is a relevant aspect as many proteases have been identified for their role in immunity but for only a few the substrate is known.

The strategy for generating EpiC2B-insensitive Pip1 is widely applicable, given knowledge of the structure of the inhibitor and methods to measure protease activity. Using a structure-based rational design, residue substitutions in Pip1 were modelled to disrupt EpiC2B binding, and engineered mutants were tested for inhibition resistance and disease response. High-accuracy interaction surface predictions, now possible with AI tools like AlphaFold-Multimer (Evans *et al.*, 2022), can streamline this process for various protease-inhibitor systems.

Pip1 belongs to the PLCP family, with a well-defined catalytic site and substrate binding groove. The Pip1–EpiC2B interaction follows a classic model, where the cystatin tripartite wedge of EpiC2B fits into Pip1's substrate binding groove (Horger and van der Hoorn, 2013). Screening only five variants identified at least one insensitive Pip1, highlighting the efficiency of this approach. Multiple mutations can confer effector insensitivity, allowing the development of gene stacks for durable resistance. Regular sequencing of pathogen populations can help select ePip1 variants to counteract emerging effector variants (Vleeshouwers *et al.*, 2011; Frantzeskakis *et al.*, 2019).

Engineering pathogen perception mechanisms using immune proteases: Pip1+

Some immune proteases contribute to pathogen perception and can therefore be engineered for broadening pathogen recognition. The Cf-2 receptor in tomato detects the complex of the Pip1 paralogue Rcr3 protease with the fungal effector Avr2 (Kruger *et al.*, 2002). It is believed that Avr2´s original target is Pip1 and hence Rcr3 acts as guarded decoy, trapping the fungus into a recognition event (van der Hoorn and Kamoun, 2008). Decoy engineering is a strategy first used for avrPphB susceptible 1 (PBS1) to become a sensor for other pathogen-produced proteases, guarded by Resistance to *Pseudomonas syringae* 5 (RPS5), the NLR immune receptors sensing PBS1 cleavage (Kim *et al.*, 2016). With a similar objective, we engineered Pip1 as well as eggplant and tobacco Rcr3 orthologs to act as co-receptor of Cf-2 (Fig. 2B). These engineered proteases were inhibited by Avr2, but between 1 and 18 amino acid changes adapted them to the Cf-2 detection system (Kourelis *et al.*, 2024).

This work has highlighted the residues that are important for protease-receptor interaction. Given the conservation of PLCP structure, it is likely that any PLCP can be turned into a Cf-2 co-receptor. This study sets the foundation for engineering the system towards broader pathogen recognition. Rcr3 is targeted not only by Avr2 but also by unrelated effectors from different pathogens (Song *et al.*, 2009; Shindo *et al.*, 2016). Modifications on the sequence of Rcr3 and its homologues should therefore enable the recognition of these effectors and the subsequent activation of the immune response. Rcr3 homologues can be found throughout Solanaceae, but the Cf-2 gene evolved in wild tomato and is present only in the *Solanum* genus (Kourelis *et al.*, 2020).

Concluding remarks

Immune proteases offer promising targets for engineering disease resistance. The strategies presented here are feasible thanks to our advanced understanding of the immune system and are therefore being applied to diverse plant immune components. The generation of inhibitor-insensitive proteases is congruent to the concept of contrasting pathogen immunosuppression for crop protection. Another example of this strategy is the 'resurrection' of plant disease resistance proteins (Contreras *et al.*, 2023). Strategies like those discussed here require minimal amino acid changes, making them compatible with genome-editing techniques such as CRISPR-Cas. This

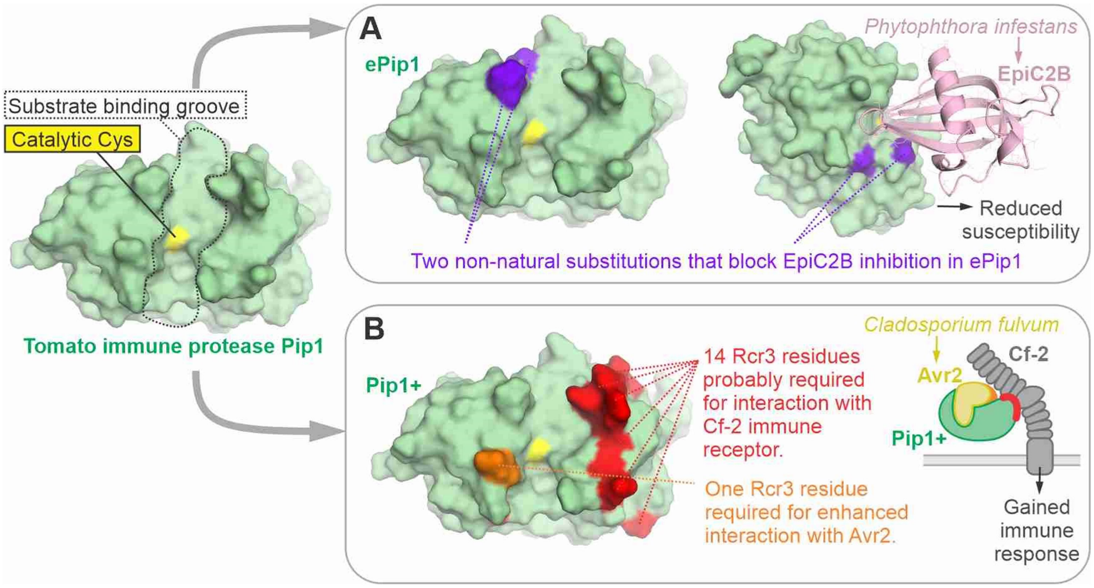

Fig. 2. Two ways of engineering *Phytophthora*-inhibited protease 1 (Pip1). (**A**) Engineering EpiC2B-insensitive Pip1 is achieved by two mutations that interfere in inhibitor binding but not with the substrate binding groove of Pip1. (**B**) Engineering Pip1 with 15 Rcr3-specific residues (red and orange) into Pip1+, results in a co-receptor triggering the hypersensitive cell death response upon co-expression with Avr2 and Cf-2.

reduces the need for transgenes, easing regulatory hurdles for crop deployment.

Rational design is accelerated by detailed biochemical knowledge, as seen in Pip1 engineering. However, alternative approaches, such as random mutagenesis or gene evolution pipelines, may also yield effective variants and even be crucials for engineering proteases insensitive to unstructured inhibitors (Zhang *et al.*, 2024). High-throughput screening systems will be essential for these strategies.

Conflict of interest

The authors have no conflicts to declare.

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References

Balakireva AV, Zamyatnin AA. 2018. Indispensable role of proteases in plant innate immunity. International Journal of Molecular Sciences **19**, 629.

Buscaill P, Sanguankiattichai N, Kaschani F, Huang J, Mooney BC, Li Y, Lyu J, Sueldo D, Kaiser M, van der Hoorn RAL. 2024. Subtilase SBT5.2 inactivates flagellin immunogenicity in the plant apoplast. Nature Communications **15**, 10431.

Contreras MP, Pai H, Selvaraj M, *et al*. 2023. Resurrection of plant disease resistance proteins via helper NLR bioengineering. Science Advances **9**, eadg3861.

Evans R, O'Neill M, Pritzel A, *et al*. 2022. Protein complex prediction with AlphaFold-Multimer. bioRxiv, 2021.2010.2004.463034.

Fescemyer HW, Sandoya GV, Gill TA, Ozkan S, Marden JH, Luthe DS. 2013. Maize toxin degrades peritrophic matrix proteins and stimulates compensatory transcriptome responses in fall armyworm midgut. Insect Biochemistry and Molecular Biology **43**, 280–291.

Frantzeskakis L, Kusch S, Panstruga R. 2019. The need for speed: compartmentalized genome evolution in filamentous phytopathogens. Molecular Plant Pathology **20**, 3–7.

Gust AA, Pruitt R, Nurnberger T. 2017. Sensing danger: key to activating plant immunity. Trends in Plant Science **22**, 779–791.

Hander T, Fernández-Fernández AD, Kumpf RP, *et al*. 2019. Damage on plants activates Ca^{2+}-dependent metacaspases for release of immunomodulatory peptides. Science **363**, eaar7486.

Horger AC, van der Hoorn RA. 2013. The structural basis of specific protease-inhibitor interactions at the plant-pathogen interface. Current Opinion in Structural Biology **23**, 842–850.

Ilyas M, Horger AC, Bozkurt TO, *et al*. 2015. Functional divergence of two secreted immune proteases of tomato. Current Biology **25**, 2300–2306.

Jumper J, Evans R, Pritzel A, *et al*. 2021. Highly accurate protein structure prediction with AlphaFold. Nature **596**, 583–589.

Kim SH, Qi D, Ashfield T, Helm M, Innes RW. 2016. Using decoys to expand the recognition specificity of a plant disease resistance protein. Science **351**, 684–687.

Kourelis J, Malik S, Mattinson O, Krauter S, Kahlon PS, Paulus JK, van der Hoorn RAL. 2020. Evolution of a guarded decoy protease and its receptor in solanaceous plants. Nature Communications **11**, 4393.

Kourelis J, Schuster M, Demir F, *et al*. 2024. Bioengineering secreted proteases converts divergent Rcr3 orthologs and paralogs into extracellular immune co-receptors. The Plant Cell **36**, 3260–3276.

Kruger J, Thomas CM, Golstein C, Dixon MS, Smoker M, Tang S, Mulder L, Jones JD. 2002. A tomato cysteine protease required for Cf-2-dependent disease resistance and suppression of autonecrosis. Science **296**, 744–747.

Liu Y, Jackson E, Liu X, Huang X, van der Hoorn RAL, Zhang Y, Li X. 2024. Proteolysis in plant immunity. The Plant Cell **36**, 3099–3115.

Marchal C, Michalopoulou VA, Zou Z, Cevik V, Sarris PF. 2022a. Show me your ID: NLR immune receptors with integrated domains in plants. Essays in Biochemistry **66**, 527–539.

Marchal C, Pai H, Kamoun S, Kourelis J. 2022b. Emerging principles in the design of bioengineered made-to-order plant immune receptors. Current Opinion in Plant Biology **70**, 102311.

Ngou BPM, Heal R, Wyler M, Schmid MW, Jones JDG. 2022. Concerted expansion and contraction of immune receptor gene repertoires in plant genomes. Nature Plants **8**, 1146–1152.

Outram MA, Figueroa M, Sperschneider J, Williams SJ, Dodds PN. 2022. Seeing is believing: Exploiting advances in structural biology to understand and engineer plant immunity. Current Opinion in Plant Biology **67**, 102210.

Pedley KF, Pandey AK, Ruck A, Lincoln LM, Whitham SA, Graham MA. 2019. Rpp1 encodes a ULP1-NBS-LRR protein that controls immunity to *Phakopsora pachyrhizi* in soybean. Molecular Plant-Microbe Interactions **32**, 120–133.

Reavy B, Bagirova S, Chichkova NV, Fedoseeva SV, Kim SH, Vartapetian AB, Taliansky ME. 2007. Caspase-resistant VirD2 protein provides enhanced gene delivery and expression in plants. Plant Cell Reports **26**, 1215–1219.

Salguero-Linares J, Coll NS. 2019. Plant proteases in the control of the hypersensitive response. Journal of Experimental Botany **70**, 2087–2095.

Schuster M, Eisele S, Armas-Egas L, Kessenbrock T, Kourelis J, Kaiser M, van der Hoorn RAL. 2024. Enhanced late blight resistance by engineering an EpiC2B-insensitive immune protease. Plant Biotechnology Journal **22**, 284–286.

Shabab M, Shindo T, Gu C, Kaschani F, Pansuriya T, Chintha R, Harzen A, Colby T, Kamoun S, van der Hoorn RA. 2008. Fungal effector protein AVR2 targets diversifying defense-related cys proteases of tomato. The Plant Cell **20**, 1169–1183.

Shindo T, Kaschani F, Yang F, *et al*. 2016. Screen of non-annotated small secreted proteins of *Pseudomonas syringae* reveals a virulence factor that inhibits tomato immune proteases. PLoS Pathogens **12**, e1005874.

Singh BK, Delgado-Baquerizo M, Egidi E, Guirado E, Leach JE, Liu H, Trivedi P. 2023. Climate change impacts on plant pathogens, food security and paths forward. Nature Reviews Microbiology **21**, 640–656.

Snoeck S, Johanndrees O, Nurnberger T, Zipfel C. 2024. Plant pattern recognition receptors: from evolutionary insight to engineering. Nature Reviews Genetics **26**, 268–278.

Song J, Win J, Tian M, Schornack S, Kaschani F, Ilyas M, van der Hoorn RA, Kamoun S. 2009. Apoplastic effectors secreted by two unrelated eukaryotic plant pathogens target the tomato defense protease Rcr3. Proceedings of the National Academy of Sciences, USA **106**, 1654–1659.

Tian M, Win J, Song J, van der Hoorn R, van der Knaap E, Kamoun S. 2006. A *Phytophthora infestans* cystatin-like protein targets a novel tomato papain-like apoplastic protease. Plant Physiology **143**, 364–377.

van der Hoorn RA, Kamoun S. 2008. From guard to decoy: a new model for perception of plant pathogen effectors. The Plant Cell **20**, 2009–2017.

van Esse HP, Reuber TL, van der Does D. 2020. Genetic modification to improve disease resistance in crops. New Phytologist **225**, 70–86.

Vleeshouwers VG, Raffaele S, Vossen JH, *et al*. 2011. Understanding and exploiting late blight resistance in the age of effectors. Annual Review of Phytopathology **49**, 507–531.

Wang S, Xing R, Wang Y, *et al*. 2021. Cleavage of a pathogen apoplastic protein by plant subtilases activates host immunity. New Phytologist **229**, 3424–3439.

Wang Y, Garrido-Oter R, Wu J, Winkelmuller TM, Agler M, Colby T, Nobori T, Kemen E, Tsuda K. 2019. Site-specific cleavage of bacterial MucD by secreted proteases mediates antibacterial resistance in Arabidopsis. Nature Communications **10**, 2853.

Xia Y, Ma Z, Qiu M, *et al*. 2020. N-glycosylation shields *Phytophthora sojae* apoplastic effector PsXEG1 from a specific host aspartic protease. Proceedings of the National Academy of Sciences, USA **117**, 27685–27693.

Zhang X, Dagvadorj B, Gao J, Molloy LM, Crean LM, Williams SJ, Rathjen JP. 2024. Identification of a key gain-of-function residue for effector binding by in vitro shuffling of barley Mla NLR genes. Molecular Plant Microbe Interactions doi: 10.1094/MPMI-11-24-0146-R.