Special Issue Editorial

Plant Issue Editorial

Proteolysis affects many processes in plant development and during stress responses, as well as being crucial in cellular protein homeostasis and recycling of resources. Beyond bulk degradation, proteases can have important signaling functions or affect cellular pathways by precise cleavage of signaling proteins. This special issue covers key research themes in the diverse but increasingly interconnected fields of programmed cell death (PCD) and plant protease activity. Future trends are also highlighted, such as accelerated substrate discovery facilitated by large-scale deposition of N-terminomic data to easily accessible databases, or better profiling using genetically encoded protease activity reporters.

Proteases encompass a diverse family of enzymes each with their own biochemical and regulatory characteristics. Although there are exceptions, they are mostly unified through their fundamental role in the hydrolysis of peptide bonds and cleavage of substrate proteins. The usual first step when talking about proteases is assigning their enzymatic class – they are classified hierarchically based on amino acid sequence similarity and chemical mechanism of catalysis. This is catalogued in the MEROPS database (Rawlings et al., 2018) with class names based on the active site amino acid or metal that performs the hydrolysis, resulting in five major groups in plants: serine (S), aspartate (A), cysteine (C), threonine (T) and metalloproteases (M) (Box 1). Papers in this issue focus on members of the pepsin-like family (A1 using the MEROPS protease nomenclature) of aspartic proteases (Soares et al., 2019a), C1p (Caseinolytic protease) serine proteases (S14) (Rodriguez-Concepcion et al., 2019), Clp (Caspase-like protease) serine proteases (S14) (Rodriguez-Concepcion et al., 2019), Clp (Caseinolytic protease) serine proteases (S14) (Rodriguez-Concepcion et al., 2019), Lon proteases (named after the long filament phenotype of bacterial mutant cells; S16) (Tsisekian et al., 2019) and metacaspases (C14B) (Klemencic and Funk, 2019). Subtilisin-like serine proteases or subtilases (S8) have also recently been reviewed (Schaller et al., 2018).

Box 1. Protease and substrate cleavage site nomenclature

Cataloguing in the MEROPS database (Rawlings et al., 2018) is based on the active site amino acid or metal that performs the hydrolysis. A curated estimate by Lallemant et al. counts 570 proteases in the Arabidopsis genome, from the most numerous serine (S) proteases (45% of the total), through cysteine (C) (25%), metalloproteases (M) (15%), aspartate (A) (11%) and, least numerous, threonine (T) (4%) proteases (Lallemant et al., 2015).

Protease substrate cleavage site nomenclature follows the form:

\[ P_4P_3P_2P_1P'_1P'_2P'_3P'_4 \]

where the downward-pointing arrow indicates the cleaved peptide bond. The most prevalent amino acids are numbered outward from this point, from the preceding ones in P positions and subsequent ones in P' positions. For example, metacaspases have a doublebasic cleavage-site pattern with arginine (R) or lysine (K) in position P_3 and P_1, respectively, and the typical substrate being valine (V) (position P_4) – R (P_3) – proline (P) (P_2) – R (P_1) (i.e. VRPR) (Klemencic and Funk, 2019).

Regulation of protease activity

Unlike many other post-translational modifications, proteolysis causes an irreversible change to its substrate proteins and so proteases need strict regulation of activity. Proteases are usually produced as pro-enzymes or zymogens. To be activated, they can perform autocatalytic cleavage in cis or trans, or be cleaved by upstream proteases from the same or completely different families. Classic examples of protease cascades from animals include blood coagulation upon injury and caspase activation during apoptosis, and Paulus and van der Hoorn (2019) have scrutinized the literature for evidence of such cascades in plants. While there are promising indications, the authors conclude that insufficient criteria have been met for known plant systems to qualify as true protease cascades. Nevertheless, given the sheer number and universal rule of signal amplification, they suggest that emerging evidence will soon change this or a new protease cascade will be identified in plants in the near future (Paulus and van der Hoorn, 2019).

Once proteases are activated, endogenous protease inhibitors can act as safety valves or have specific signaling roles by modulating protease activity (Grosse-Holz and van der Hoorn, 2016). Cohen et al. (2019) examine the serpins, a widely conserved class of inhibitors, and how they exploit protease activity to covalently bind and distort protease structure. Although originally named from ‘serine protease inhibitor’, serpins...
can inhibit a wide range of proteases including cysteine proteases. Irreversible or reversible inhibition within seconds of contact with a serpin can regulate biological functions, as in the case of set-point control of the pro-cell death protease RESPONSIVE TO DESICCATION 21 (RD21) (Lampl et al., 2013). Interestingly, serpins can also act as chaperones in other contexts, for example to increase beta-amylase activity in barley grains (Cohen et al., 2019).

Protease activity can also be controlled by physically separating the enzyme from its substrates through subcellular localization. Remobilization of phytaspase, a subtilase with aspartate specificity, from the apoplast to the cell interior occurs under hypersensitive response (HR)-like cell death triggered by tobacco mosaic virus infection (Chichkova et al., 2010; Salguero-Linares and Coll, 2019). In an intriguing Viewpoint article, Trusova et al. (2019) put forward the hypothesis that this is caused by clathrin-dependent endocytosis, at least during oxidative stress-triggered cell death. Furthermore, proteases are present in all cellular compartments and can exert specific functions in these in isolation from the rest of the cell. Rodriguez-Concepcion et al. (2019) review the existing literature on the chloroplast-localized Clp complexes and substrates. While the authors put forward clear rules as to what constitutes a confirmed Clp substrate, definitive proof is hard to come by and ideally involves both in vivo and in vitro confirmation.

**Protease substrates**

For protein homeostasis-related proteases (proteasome and ATP-dependent proteases such as Clp, Fsh and Lon) substrate identification remains a challenging task. Often because of severe and pleiotropic mutant phenotypes in combination with a lack of clear cleavage site (Box 1) motifs – instead of cleaving a substrate once, resulting in a defined cleavage site motif, the enzyme can cleave at many sites in the substrate with lesser site specificity and so it is hard to assign a substrate through prediction of cleavage sites or matching to a known protease specificity profile. To obtain evidence for bona fide substrates, multiple follow-up experiments are required with substrate trapping being a particularly promising way forward (Rei Liao and van Wijk, 2019). In a substantial advance for serine and cysteine proteases, a proof-of-concept was recently established for substrate trapping by replacement of the active site cysteine or serine with the non-native amino acid 2,3-diaminopropionic acid (DAP) (Huguenin-Dezot et al., 2019). Although only performed with recombinant protein, the tobacco etch virus (TEV) protease and its native substrate were successfully trapped through a covalent amide bond. This opens up new routes for the identification of protease substrates from complex protein extracts.

More established methods for proteomics-scale protease substrate discovery include terminal amine isotopic labeling of substrates (TAILS) and combined fractional diagonal chromatography (COFRADIC) (reviewed by Demir et al., 2018; Perrar et al., 2019). These rely on the enrichment and identification by mass spectrometry of N- or C-terminal start positions at the original protein terminus or internal proteolysis sites, hence the term N- or C-terminomics (or degradomics). In fact, most studies in plants have focused on applications other than protease substrate discovery, including alternative translation initiation, post-translational modification of the N-terminus (e.g. by acetylation), and N-end rule substrate discovery (Perrar et al., 2019). Some relate to protein homeostasis, but all emphasize the importance of the N-terminal amino acid for protein function.

Some studies challenge the idea that proteases must exert their function through substrate cleavage and these need to be taken into consideration when trying to uncover protease substrates. Fsh proteins are presumably inactive Fsh proteases as their active sites are mutated. In a comprehensive analysis of FshI mutant phenotypes, Mishra et al. (2019) have found that inactive FshI homologs affect chloroplast function and plant development. Furthermore, in the right conditions, some proteases can ligate peptides, for example in the production of cyclic peptides by asparaginyl endopeptidases such as legumain and butelase (Zauner et al., 2018; James et al., 2019). Also, the presence of smaller molecular weight protein products is not necessarily the result of proteolysis. They might well result from alternative splicing or alternative translation initiation (see Willems et al., 2017; Perrar et al., 2019).

**PCD and cell survival**

Salguero-Linares and Coll (2019) review the roles of proteases, including papain-like cysteine proteases, metacaspases, vacuolar-processing enzymes, proteasomal subunits and subtilases in HR-type cell death. HR is triggered upon pathogen infection and has been known for over a hundred years, receiving significant attention for its role in pathogen resistance. Although the authors conclude that mechanistic insight into the process of cell death remains scarce, it is hoped that concerted action spanning the fields of both cell death and protease activity will accelerate this process in the near future (Salguero-Linares and Coll, 2019).

Andrade Buono et al. (2019) review the roles and functions of proteases during developmentally controlled programmed cell death (dPCD). Though several proteases and protease activities have been implicated in the regulation of cell death and corpse degradation, again little mechanistic insight into the precise role and targets of proteases during dPCD processes is available (Andrade Buono et al., 2019). A special type of dPCD occurring during the self-incompatibility (SI) response in field poppy has been reviewed by Wang et al. (2019). A self-recognition-based signaling network leads to arrested growth and finally PCD of self-pollen. Pollen SI-PCD features prominent cytosolic acidification and caspase-like protease activity. Despite substantial efforts, the SI-PCD proteases responsible for this activity remain to be identified (Wang et al., 2019).

Escamez et al. (2019) report on the discovery of the novel extracellular peptide Kratos in the context of xylem cell death. Kratos is not involved in differentiation or PCD in the xylem tracheary elements, but is important for the protection of neighboring non-tracheary element cells from ectopic cell death.
death. This ectopic cell death occurs in metascape9 mutants and when autophagy is up-regulated in tracheary elements. Likely acting downstream of autophagy, Kratos acted to protect neighboring non-tracheary element cells from ectopic cell death during xylem differentiation. Furthermore, Kratos had an ameliorative effect on cell death in stresses other than xylem cell death, such as wounding and cell death triggered by reactive oxygen species.

**Functions other than PCD**

Soares *et al.* (2019a) note the remarkable expansion of atypical aspartic proteases in plants, which has led to a diversity of specialized functions. These atypical and nucellin-like aspartic proteases vary widely in their enzymatic properties and subcellular localizations, including activity at higher-than-acidic pH, incomplete pepstatin inhibition, and divergence from hydrophobic, aliphatic or aromatic amino acids in the P1 and P1' position (e.g. Phe–Phe) (Soares *et al.*, 2019a). The authors also discuss the various functions of aspartic proteases and additional research on the novel ATYPICAL ASPARTIC PROTEASE IN ROOTS 1 (ASPR1). The biochemical characteristics of ASPR1 were found to be surprisingly similar to fungal aspartic proteases. Expression analysis and gain- and loss-of-function experiments showed that ASPR1 has a role in primary root elongation and lateral root formation (Soares *et al.*, 2019b).

Tornkvist *et al.* (2019) describe possible ways in which proteases and proteostasis might contribute to nitrogen use efficiency (NUE). Improving NUE is an important target for crop breeding, as run-off from excess nitrogen fertilization must be limited to enhance sustainable and environment-friendly farming. Digestive proteolysis mediated by the proteasome and autophagy would be expected to contribute to NUE. Symbiotic nitrogen fixation and nodule formation are in certain instances regulated by proteases. For less-explored scenarios, but drawing from existing examples, the authors propose situations in which proteases could generate nitrate-sensing peptide signals or alter peptide receptors, influence root cap sloughing, or directly regulate NO3- or NH3-transporters. They conclude with a familiar call that to improve our understanding we need to increase our knowledge of protease–substrate and protease–protease interactions, and that improvement in NUE seems feasible through manipulation of proteolytic pathways (Tornkvist *et al.*, 2019).

Many C1A cysteine proteases, including the cathepsins, have typically been associated with nutrient recycling. Gomez-Sanchez *et al.* (2019) found four genes to be up-regulated in barley leaves upon drought stress. Knock-down lines for two of these genes, HvPap-1 and HvPap-19, led to changes in leaf cuticle thickness and stomatal pore area, and photosystem efficiency and protein homeostasis were much less affected than in drought-stressed wild-type plants. Unexpectedly, stress hormone levels were altered and the changes in cuticle thickness and stomatal pore area had advantageous effects on leaf defense against fungi and mites (Gomez-Sanchez *et al.*, 2019).

Protein homeostasis or proteostasis is intricately linked to protease activity and programmed cell death (Minina *et al.*, 2017; Ustun *et al.*, 2017; Have *et al.*, 2018). Increasing interconnection between the protease field and the field of N-end rule-regulated proteostasis is to be expected. The N-end rule pathways recognize degradation signals that mainly depend on the N-terminal amino acid residue of a protein and determine its half-life. Protease–generated substrate fragments might well be subject to the N-end rule (Dissmeyer *et al.*, 2018; Millar *et al.*, 2019).

**Challenges and future perspectives**

In comparison to animal or human studies, surprisingly few definite protease substrates have been identified in plants. The above-mentioned proteomic techniques (Demir *et al.*, 2018) usually deliver long candidate lists from which few substrates are subsequently validated, and at present most substrates are still found on a gene-by-gene protease-substrate basis through logical deduction. Nevertheless, peptide signaling has proven to be a particularly strong area of substrate discovery, partly because there is an obvious need to liberate small signaling peptides from their precursor proteins (Wrzaczek *et al.*, 2015; Besho-Uehara *et al.*, 2016; Schardon *et al.*, 2016). Elucidating the relevance and role (e.g. activation, deactivation or neo-functionalization) of substrate cleavage will remain an arduous task.

Substrate discovery should accelerate in the future (Savickas and Auf dem Keller, 2017; Perrar *et al.*, 2019). It would definitely help if there was increased interest in substrate cleavage events and the proteases responsible, and to recognize substrate cleavage as a post-translational modification (PTM) on a par with, for example, phosphorylation (Millar *et al.*, 2019). Deposition of large-scale N-terminomic data to easily accessible databases, such as the Plant PTM viewer, could be one way to reach this goal (Willems *et al.*, 2018, Preprint).

Powerful new tools are increasingly becoming available for probing protease activity in plants or plant protein extracts, and these include (mainly) chemical tools for activity-based protein profiling (ABPP) (Morimoto and van der Hoorn, 2016). Far less explored in plants is a new generation of genetically encoded protease activity reporters (Fernández–Fernández *et al.*, 2019), the majority of which have so far only been used in mammalian studies. These will help us gain a better understanding of protease activation and mode of action in plants (van der Hoorn and Rivas, 2018; Klemcnic and Funk, 2019). As protease activity and substrate cleavage go hand in hand, it is crucial to understand the activity of the protease of interest both in vitro and in vivo to discover and make sense of the substrate cleavage event.

Mechanistic insight into the initiation and execution of cell death remains a bottleneck (Andrade Bueno *et al.*, 2019; Salguero-Linares and Coll, 2019; Wang *et al.*, 2019). The discovery of new modalities of cell death in plants, such as ferptosis (Dangol *et al.*, 2019), and potential cell survival strategies (Escamez *et al.*, 2019), will add new layers of complexity. Identifying the proteases responsible in this context will be an important target for the near future.
Acknowledgments

The authors would like to thank all participants and organizers of the Plant Protease and Programmed Cell Death meeting that took place in Ghent, Belgium in 2018 and contributors to this special issue. We apologize for not citing all relevant research and hope that the reader will find their way to the relevant studies in the special issue papers.

Keywords: Activity-based protein profiling (ABPP), development, peptide signaling, post-translational modification, programmed cell death, protease activity reporters, proteases, protein homeostasis, proteolysis, stress.

Simon Stael1,2,3,4,*, Frank Van Breusegem1,2, Kris Gevaert3,4 and Moritz K Nowack1,2

1Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium
2VIB-UGent Center for Plant Systems Biology, 9052 Ghent, Belgium
3Department of Biomolecular Medicine, Ghent University, 9000 Ghent, Belgium
4VIB-UGent Center for Medical Biotechnology, 9000 Ghent, Belgium

*Correspondence: Simon.Stael@psb.vib-ugent.be

References

Andrade Buono R, Hudecek R, Nowack MK. 2019. The roles of proteases during developmental programmed cell death in plants. Journal of Experimental Botany 70, 2097–2112.

Bessho-Uehara K, Wang DR, Furuta T, et al. 2016. Loss of function at RAE2, a previously unidentified EPFL, is required for aveniness in cultivated Asian rice. Proceedings of the National Academy of Sciences, USA 113, 8969–8974.

Chichkov NV, Shaw J, Galullina RA, et al. 2010. Phytaspase, a relcausible cell death promoting plant protease with caspase specificity. The EMBO Journal 29, 1149–1161.

Cohen M, Davydov O, Fluur R. 2019. Plant serpin protease inhibitors: specificity and duality of function. Journal of Experimental Botany 70, 2077–2085.

Dangol S, Chen Y, Hwang BK, Jwa NS. 2019. Iron- and reactive oxygen species-dependent ferroptotic cell death in rice-magnaporthe oryzea interactions. The Plant Cell 31, 189–209.

Demir F, Niedermaier S, Villamor JG, Huesgen PF. 2018. Quantitative proteomics in plant protease substrate identification. New Phytologist 218, 936–943.

Dissmeyer N, Rivas S, Graciet E. 2018. Life and death of proteins after protease cleavage: protein degradation by the N-end rule pathway. New Phytologist 218, 929–935.

Escamez S, Stael S, Vainonen JP, Willems P, Jin H, Kimura S, Van Breusegem F, Gevaert K, Wrzaczek M, Tuominen H. 2019. Extracellular peptide Kratos restricts cell death during vascular development and stress in Arabidopsis. Journal of Experimental Botany 70, 2199–2210.

Fernández-Fernández AD, van der Hoorn RAL, Gevaert K, Van Breusegem F, Stael S. 2019. Caught green-handed: methods for in vivo detection and visualization of protease activity. Journal of Experimental Botany 70, 2125–2141.

Gomez-Sanchez A, Gonzalez-Melendi P, Estrella Santamaria M, Arbona V, Lopez-Gonzalez A, Garcia A, Hensel G, Kumlehn J, Martinez M, Diaz I. 2019. Repression of drought-induced cysteine-protease genes alters barley leaf structure and responses to abiotic and biotic stresses. Journal of Experimental Botany 70, 2143–2155.

Grosse-Holz FM, van der Hoorn RA. 2016. Juggling jobs: roles and mechanisms of multifunctional protease inhibitors in plants. New Phytologist 210, 794–807.

Havé M, Bailiau T, Cottyn-Boitte B, et al. 2018. Increases in activity of proteasome and papain-like cysteine protease in Arabidopsis autophagy mutants: back-up compensatory effect or cell-death promoting effect? Journal of Experimental Botany 69, 1369–1385.

Huguenin-Dezet N, Alonzo DA, Heberlig GW, Mahesh M, Nguyen DP, Dornan MH, Boddy CN, Schmeing TM, Chin JW. 2019. Trapping biosynthetic acyl-enzyme intermediates with encoded 2,3-diaminopropionic acid. Nature 565, 112–117.

James AM, Haywood J, Leroux J, et al. 2019. The macrocytolyzing protease butelase 1 remains auto-catalytic and reveals the structural basis for ligase activity. The Plant Journal.

Klemencic M, Funk C. 2019. Variations on a theme: structural diversity in metacaspases. Journal of Experimental Botany 70, 2039–2047.

Lallemand J, Bouché F, Desiron C, Stautemas J, de Lemos Esteves F, Pérelleux C, Tocquin P. 2015. Extracellular peptidase hunting for improvement of protein production in plant cells and roots. Frontiers in Plant Science 6, 37.

Lamp L, Alkan N, Davydov O, Fluur R. 2013. Set-point control of RD21 protease activity by AtSerpin1 controls death in Arabidopsis. The Plant Journal 74, 498–510.

Millar AH, Heazlewood JL, Giglione C, Holdsworth MJ, Bachmair A, Schulze WX. 2019. The scope, functions, and dynamics of posttranslational protein modifications. Annual Review of Plant Biology.

Minina EA, Moschou PN, Bozhkov PV. 2017. Limited and digestive proteolysis: crosstalk between evolutionary conserved pathways. New Phytologist 215, 958–964.

Mishra LS, Mielke K, Wagner R, Funk C. 2019. Reduced expression of the proteolytically inactive FtsH members has impacts on the Darwinian fitness of Arabidopsis thaliana. Journal of Experimental Botany 70, 2173–2184.

Morimoto K, van der Hoorn RA. 2016. The Increasing Impact of Activity-Based Protein Profiling in Plant Science. Plant & Cell Physiology 57, 446–461.

Paulus JK, van der Hoorn RAL. 2019. Do proteolytic cascades exist in plants? Journal of Experimental Botany 70, 1997–2002.

Perrar A, Dissmeyer N, Huesgen P. 2019. New beginnings and new ends – methods for large-scale characterization of protein termini and their use in plant biology. Journal of Experimental Botany 70, 2021–2038.

Rawlings ND, Barrett AJ, Thomas PD, Huang X, Bateman A, Finn RD. 2018. The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. Nucleic Acids Research 46, D624–D632.

Rei Liao JY, van Wijk KJ. 2019. Discovery of AAA+ protease substrates through trapping approaches. Trends in Biochemical Sciences.

Rodriguez-Concepcion M, D’Andrea L, Pulido P. 2019. Control of plastidial metabolism by the Cip protease complex. Journal of Experimental Botany 70, 2049–2058.

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

Savickas S, Auf dem Keller U. 2017. Targeted degradomics in protein terminomics and protease substrate discovery. Biological Chemistry 399, 47–54.

Schaller A, Stintzi A, Rivas S, et al. 2018. From structure to function - a family portrait of plant subtilases. New Phytologist 218, 901–915.

Schardon K, Hohl M, Graff L, Pfannstiel J, Schulze W, Stintzi A, Schardin C. 2016. Juggling jobs: roles and mechanisms of multifunctional protease inhibitors in plants. New Phytologist 210, 794–807.

Soares A, Niedermaier S, Faro R, Loos A, Manadas B, Faro C, Huesgen PF, Cheung AY, Simões I. 2019b. An atypical aspartic protease modulates lateral root development in Arabidopsis. Journal of Experimental Botany 70, 2157–2171.

Soares A, Ribeiro Carlton SM, Simões I. 2019a. Atypical and nucellin-like aspartic proteases: emerging players in plant developmental processes and stress responses. Journal of Experimental Botany 70, 2059–2076.

Tornkvist A, Liu C, Moschou PN. 2019. Proteolysis and nitrogen: emerging insights. Journal of Experimental Botany 70, 2009–2019.
Trusova SV, Golyshev SA, Chichkova NV, Vartapetian AB. 2019. Sometimes they come back: endocytosis provides localization dynamics of a subtilase in cells committed to cell death. Journal of Experimental Botany 70. 2003–2007.

Tsitsekian D, Daras G, Alatzas A, Templalexis D, Hatzopoulos P, Rigas S. 2019. Comprehensive analysis of Lon proteases in plants highlights independent gene duplication events. Journal of Experimental Botany 70. 2185–2197.

Üstün S, Hafren A, Hofius D. 2017. Autophagy as a mediator of life and death in plants. Current Opinion in Plant Biology 40. 122–130.

van der Hoorn RAL, Rivas S. 2018. Unravelling the mode of action of plant proteases. New Phytologist 218. 879–881.

Wang L, Lin Z, Triviño M, Nowack MK, Franklin-Tong VE, Bosch M. 2019. Self-incompatibility in Papaver pollen: programmed cell death in an acidic environment. Journal of Experimental Botany 70. 2113–2123.

Willems P, Horne A, Goormachtig S, De Smet I, Botzki A, Van Breusegem F, Gevaert K. 2018. The Plant PTM Viewer, a central resource exploring plant protein modifications. from site-seeing to protein function. bioRxiv 415802 [Preprint].

Willems P, Ndah E, Jonckheere V, Stael S, Sticker A, Martens L, Van Breusegem F, Gevaert K, Van Damme P. 2017. N-terminal proteomics assisted profiling of the unexplored translation initiation landscape in Arabidopsis thaliana. Molecular & Cellular Proteomics 16. 1064–1080.

Wrzaczek M, Vainonen JP, Stael S, et al. 2015. GRIM REAPER peptide binds to receptor kinase PRK5 to trigger cell death in Arabidopsis. The EMBO Journal 34. 55–66.

Zauner FB, Elsässer B, Dall E, Cabrele C, Brandstetter H. 2018. Structural analyses of Arabidopsis thaliana legumain γ reveal differential recognition and processing of proteolysis and ligation substrates. The Journal of Biological Chemistry 293. 8934–8946.