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Special Issue Editorial

Plant proteases and programmed cell death

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), Clp (Caseinolytic protease) serine proteases (S14) (Rodriguez-Concepcion *et al.*, 2019), Lon proteases (named after the long filament phenotype of bacterial mutant cells; S16) (Tsitsekian *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).

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

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 Lallemand et al. counts 570 proteases in the Arabidopsis genome, from the most numerous serine (S) proteases (45% of the total), through cysteine (C) (25%), metallo (M) (15%), aspartate (A) (11%) and, least numerous, threonine (T) (4%) proteases (Lallemand *et al.*, 2015).

Protease substrate cleavage site nomenclature follows the form:

$P_4P_3P_2P_1{\downarrow}P'_1P'_2P'_3P'_4$

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).

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, definite 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, Ftsh 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. Ftshi proteins are presumably inactive Ftsh proteases as their active sites are mutated. In a comprehensive analysis of Ftshi mutant phenotypes, Mishra *et al.* (2019) have found that inactive FtsH 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. This ectopic cell death occurs in *metacaspase9* 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 peptidic signals or alter peptide receptors, influence root cap sloughing, or directly regulate NO_3- or NH_4-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; Bessho-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; Klemencic 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 Buono *et al.*, 2019; Salguero-Linares and Coll, 2019; Wang *et al.*, 2019). The discovery of new modalities of cell death in plants, such as ferroptosis (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.

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

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