Commentary

Unravelling the mode of action of plant proteases

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Higher plant genomes encode over 700 proteases that irreversibly regulate protein fate. This expansion in the plant protease repertoire is coherent with proteases being involved in the regulation of virtually every plant process. In agreement, the activity, substrates and biological functions of proteases are extensively investigated by researchers in all fields in plant biology.

In 2016, the 3rd International Conference on Plant Proteases entitled *"Plant Proteases: from roles in life and death to understanding regulation and action"* was held in Oxford with the support of *New Phytologist.* Some of the leading scientists attending this conference contributed to this Special Issue in which they present novel concepts, approaches and standards in plant protease research.

Papers in this Special Issue underline the close connections between protease activity and the regulation of cell death-related processes. Indeed, proteases are central regulators of various forms of developmental and defence-related programmed cell death (PCD), e.g. during plant-microbe interactions, cell patterning, autophagy, senescence, xylem development or seed germination (Van der Hoorn, 2008). In animal cells, caspases are cysteine proteases that play central roles in inflammation and apoptosis. Fungi, plants and algae do not have caspases but do have proteases with caspase-like activities and proteins with structural homology to caspases such as metacaspases (Uren et al., 2000). In plants, activation of caspase-3-like activity has been shown during ER-stress-induced PCD, a form of PCD that takes place when protein homeostasis in the ER is perturbed (Williams et al., 2014). In their paper, Cai *et al.* (pp. xx-xx) show that cathepsin B and the proteasome subunit PBA1 contribute to caspase-3-like activity during ER stress. PCD triggered by ER-stress is positively regulated by cathepsin B and

repressed by PBA1, although the *in vivo* substrates of these proteases remain to be identified. Another interesting example of a protease comprising a pseudo-caspase and an active caspase-like fold is separase (Lin et al., 2016). This modular protease contains a so-called 'death domain' (DD) in its pseudo-caspase fold. In their review article, Liu and Moschou (pp. xx-xx), discuss the modular structure of Arabidopsis separase as well as its dual role in the control of auxin distribution and thus of root growth and gravitropism. Indeed, separase not only functions in the secretory sorting and localization of the auxin carrier PIN2 through cleavage of unknown substrates (Moschou et al., 2013), but also regulates PIN2 sorting through non-proteolytic promotion of microtubule stability (Moschou et al., 2016).

Plant metacaspases are typically classified according to the presence (type I) or absence (type II) of an N-terminal prodomain that is predicted to negatively regulate protease activity. In this Special Issue, Lema-Asqui *et al.* (pp. xx-xx) identify the protease inhibitor AtSerpin1 as a negative regulator of the self-processing and cell death-promoting activity of AtMC1, an Arabidopsis type I metacaspase that promotes the hypersensitive response in response to bacteria. As the regulatory modes of type I and II metacaspases are uncovered, the development of sequencing technologies gives access to genes encoding other types of metacaspases in other organisms. For example, through a bioinformatic analysis, Klemenčič and Funk (pp. xx-xx) identified a type III metacaspase, named GtMC2, in the cryptophyte *Guillardia theta*. Biochemical characterization shows that GcMC2 is an active endopeptidase that presents two calcium-biding sites and is functionally related to plant type I metacaspases.

Besides metacaspases, also subtilases (SBTs) have attraced considerable attention. In a Tansley Review article, Schaller *et al*. (pp. xx-xx) provide a comprehensive overview of the structure, organisation and physiological roles of the largest family of plant proteases: the subtilase (SBT) family of serine proteases. During evolution, functional diversification led to the expansion of the SBT protein family and resulted in the acquisition of a large repertoire of plant-specific developmental functions including embryogenesis, seed development and germination, cuticle formation and epidermal patterning, xylem development, organ abscission or senescence. In addition, SBT proteins are also involved in the regulation of biotic and abiotic stress responses. In this Special Issue, Beloshistov *et al*. (pp. xx-xx) identify a new role of phytaspases. Phytaspases are aspartate-specific proteases of the SBT family, previously involved in triggering PCD in response to biotic and abiotic insults (Chichkova et al., 2010). The finding that phytaspases are able to process prosystemin, a precursor of the tomato wound hormone systemin, highlights the importance of SBT proteases for systemic wound signaling

and provides further proof of the connections between cell death-related protease activity and defence signalling. Liu and Moschou (pp. xx-xx) also review the particular case of SBT5.2 that was first described as a negative regulator of stomatal density under high CO_2 conditions through proteolytic cleavage of Epidermal Patterning Factor 2 (EPF2) in the apoplast (Engineer et al., 2014). Alternative splicing of the *SBT5.2* gene gives rise to the non-proteolytically active isoform SBT5.2b. SBT5.2b attenuates the defence-related hypersensitive cell death through nuclear exclusion of the transcription factor MYB30 that is retained by SBT5.2b in endosomal vesicles (Serrano et al., 2016).

An additional major player in the control of protein stability is the ubiquitin proteasome system (UPS). The N-end rule pathway is a subset of the UPS that relates the *in vivo* half life of a protein to the nature and post-translational modification of its N-terminal amino acid residue. Proteases other than the proteasome itself play an important role in the generation of N-end rule substrates as endoproteolytic cleavage of a pre-proprotein results in the exposure of a new N-terminal residue that, following recognition by the N-end rule pathway, may lead to proteasomal degradation of the target. In a Tansley insight article, Dissmeyer *et al*. (pp. xx-xx) discuss recently discovered functions of the plant N-end rule pathway and the important role of proteases in the generation of N-end rule substrates. Technical challenges around the identification of N-end rule substrates are also discussed. The importance of substrate identification in understanding protease function extends well beyond the N-end rule pathway and, despite the high number of proteases described, very few of their substrates have been identified to date. The review article by Demir *et al*. (pp. xx-xx) focuses on sensitive, novel mass spectrometry-based techniques that are being used for substrate uncover protease susbtrates and function. Reviewed techniques include quantitative proteomics for candidate substrate identification, metabolic stable isotope labeling in pulse or pulse-chase assays for measure *in vivo* protein turnover and N- and C-terminomics for large scale identification of cleavage sites. The use of N-terminomics is a powerful tool for N-end rule substrate identification. For example, Zhang *et al*. (pp. xx-xx) used terminal amine isotopic labelling of substrates with tandem mass tags (TMT-TAILS) for relative quantification of N-terminal peptides in the Arabidopsis N-end rule mutant *prt6* to investigate the impact of the N-end rule pathway on the proteome of etiolated seedlings. This study identifies a novel role for PRT6 in regulating the abundance of seed storage proteins and proteases that optimize reserve mobilization during the transition from seed to seedling.

Vacuolar processing enzymes (VPEs) are yet another important caspase-related class of proteases involved in PCD regulation. VPEs have been implicated in PCD regulation in both immunity and plant development. The article of Radchuk et al. (pp. xx-xx) demonstrates the role of VPE4 in PCD in the pericarp during seed development in barley seeds to make space for the growing embryo. The role of VPE4 in pericarp PCD is distinct but somewhat similar to the role of Arabidopsis δVPE in the regulation of seed coat PCD (Nakaune et al., 2005). VPEs are sometimes also called asparaginyl endopeptidase (AEPs) and these VPEs/AEPs play an intriguing role in creation of novel proteins, again often in seeds. In a Tansley insight article, James et al., (pp. xx-xx) discusses the role of AEPs in the evolution and production a bizarre diversity of cyclic peptides in plants. The ability of proteases to not only cut but also create a peptide bond expands their role beyond proteolysis into the creation of novel proteins. This observation highlights a recurring theme in protease research: proteases may also fulfill their biological roles without cleaving peptide bonds.

Overall, this Special Issue illustrates the great diversity of biological questions investigated by researchers in the protease field. And yet, they all face common challenges, often on the identification of biologically relevant substrates. In 2018, the 4th Plant Protease and PCD Symposium will be held in Gent (Belgium). Supported by *New Phytologist*, it will bring together again the plant protease and cell death community. We look forward to this meeting that, we anticipate, will provide exciting discussions around the latest developments in this field.

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