

Plant Proteolysis in Development: Insights and Functions



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Abstract Plant proteolysis is an important process through which proteome quality and quantity surveillance is accomplished. Proteases regulate protein turnover and expand their functional diversity. Proteolysis is mostly known as a means to remove proteins but may also result in the production of new shorter proteins. Hence, proteolysis is directly linked with many aspects of the plant’s lifecycle. Here, we provide an overview of selected examples of the major proteolytic pathways known, digestive (autophagy, proteasome, ubiquitin-like pathways and N-end rule) and limited proteolysis in development.

Keywords Autophagy, Development, NEDD, N-end rule, Proteases, Proteolysis, SUMO

1 Introduction

In plants, cellular and extracellular proteomes show remarkable functional flexibility, a result of different posttranslational fates, including a broad range of covalent modifications (Liu et al. 2020). Almost all proteins, regardless of their lifetime, biochemical properties and cellular function, are sooner or later subjected to proteolysis (Liu and Moschou 2018). The term “proteolysis” is erroneously associated usually with protein end-point degradation and thus loss-of-function. However, proteolysis is like a Swiss-knife and has many trades. Indeed, each protein thus has multiple proteolytic routes on which to embark, with proteolysis outcomes varying between two extremes: either complete degradation (digestive proteolysis) or specific cleavage of the polypeptide chain at one or a few sites (limited proteolysis). While the outcome of digestive proteolysis is usually protein destruction and functional loss, limited proteolysis can additionally lead to the maturation of proteins or peptides and gain- or switch-of-function (Liu and Moschou 2018). The substrate and products of many proteolytic modules are known; however, the corresponding protease remains unidentified. By contrast, many proteases have unknown substrates (orphan proteases).

In this chapter, we discuss proteolytic aspects with direct links to development. We also provide an overview of biological pathways that utilize specific proteolytic cleavage as a mechanism for tuning their outcomes. Due to the immense amount of data on the chapter’s topic, our discussion is succinct and by no means exhaustive. We thus occasionally refer the interested reader to excellent reviews that provide deeper insights.

1.1 *The Basics of Limited and Digestive Proteolysis*

While all proteins give in to proteolytic degradation, the proteome fraction subjected to limited proteolysis remains elusive. Often regarded as a mere degradative mechanism in the destruction of proteins or turnover in maintaining physiological homeostasis, recent research in the field of degradomics (i.e. the high-throughput study, usually by proteomics, that can detect proteolysis products and even cleavage sites) has led to the recognition of two main yet unexpected concepts. First, that targeted proteolytic cleavage events by a wide repertoire of proteases are pivotal regulators of most, if not all, developmental processes. Second, an unexpected *in vivo* abundance of stable cleaved proteins revealed pervasive, functionally relevant protein processing.

Below, we succinctly describe the two main proteolytic branches. As of note, as the C-end rule pathway has only recently emerged as a potential proteolytic branch and is as yet unknown in plants, we refer interest readers to, e.g., Lin et al. (2018).

1.2 *Digestive Proteolysis*

1.2.1 *Autophagy*

Autophagy carries out digestive proteolysis and usually requires endoproteases for full functionality. Autophagic protein degradation takes place in the vacuole and can be either bulk or selective, i.e. when certain cellular components are preferentially targeted for destruction (Fig. 1a; (Minina et al. 2017a)). Selective autophagy is determined by the interaction of ATG8 with specific cargoes regardless of their size. Readers may refer to Marshall and Vierstra (2018), which is an overview regarding selective autophagy. As of note, the repertoire of autophagy extends beyond proteolytic degradation; it also digests nucleic acids, lipids and carbohydrates but here we will focus on proteolysis.

Autophagic degradation is carried out via recognition of substrates by specific adaptors and their sequestration into a double-membrane vesicle, the autophagosome, which is then delivered to and degraded in the vacuole (Minina et al. 2017a). Autophagy-related (ATG) proteins are posttranslationally activated and modulate autophagosome biogenesis ((Marshall et al. 2019) and the references therein). Interestingly, autophagy is regulated by limited proteolysis that takes place in the early stages of the autophagy pathway. In particular, the small ubiquitin (Ub)-like protein ATG8 is cleaved C-terminally by the cysteine protease ATG4 at a highly conserved Gly and the exposed C-terminal glycine is conjugated with the growing autophagosomal membrane (Yoshimoto et al. 2004; Pérez-Pérez et al. 2021). This residue coincides with the penultimate residue in yeast, while in most ATG8s this conserved Gly does not occupy this position. ATG8 is then released from the mature autophagosome membrane by the same ATG4 protease (Pérez-Pérez et al. 2021).

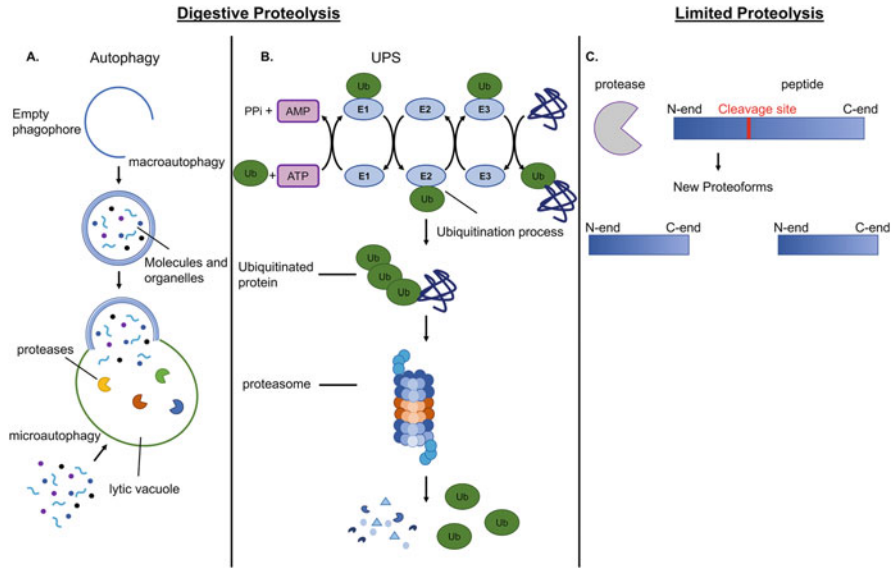


Fig. 1 Schematic representation of the major pathways in digestive and limited proteolysis. (a) Autophagy: Molecules and organelles are either enclosed in autophagosomes (macro-autophagy) or enter directly the lytic vacuole (micro-autophagy). (b) Ubiquitin-Proteasome System: Proteins are selectively ubiquitinated and recognized by proteasome leading to full degradation. (c) Limited proteolysis: Proteases recognize and cleave their protein-targets, creating new proteoforms with different properties such as new interactions or subcellular localization. Figure was created using BioRender (<https://biorender.com>)

1.2.2 Proteasome

Unlike autophagy, the Ub-proteasome system (UPS) is almost always selective degrading only Ub-tagged proteins. In the UPS pathways, Ub covalently conjugates to substrate lysines (K) through activating (E1), conjugating (E2) and ligating (E3) enzymes (Sadanandom et al. 2012; Santner and Estelle 2010; Spoel et al. 2009; Eckardt 2001; Emenecker et al. 2020; Lin et al. 2020). Ub is first activated by E1 and then transferred onto an E2 conjugating enzyme. Subsequently, E3 Ub-ligases interact simultaneously with a Ub-loaded E2 and the substrate and mediate the formation of isopeptide bonds between the Ub C-terminus and the acceptor K. Depending on the Ub modification nature (e.g. K48 or K63), Ub-carrying proteins have different fates, two prominent examples of which are degradation by the UPS (K48-linked Ub chains) and endocytosis from the plasma membrane to the vacuole (K63-linked chains) (Schwechheimer 2018). The single-subunit E3 ligases include proteins containing a conserved REALLY INTERESTING NEW GENE (RING) domain, a U-box domain, or a Homologous to the E6-AP Carboxyl Terminus (HECT) domain that mediates interaction with the E2-Ub.

1.2.3 Ubiquitin-Like Pathways

Other posttranslational modifications (PTMs) may associate with digestive proteolytic pathways. For example, an analogous enzymatic cascade to Ub is protein tagging via isopeptide bonds with SUMO (small Ub-like modifier) (Srivastava et al. 2016; Yates et al. 2016a, b). Other Ub-like modifiers (UBLs) include NEDD8 (NEURAL PRECURSOR CELL EXPRESSED, DEVELOPMENTALLY DOWN-REGULATED 8) or RUB1 (related to Ub 1) (Schwechheimer 2018), URM1 (Ub-related modifier-1) (Wang et al. 2019), ATG8/12 (autophagy 8/12), MUB (membrane-anchored Ub) (Dowil et al. 2011), UFM1 (Ub-fold modifier-1) (Sasakawa et al. 2006) and HUB1 (homology to Ub-1) (Downes and Vierstra 2005). Like in the case of Ub, UBL conjugation generally depends on an E1-E2-E3 cascade and targets the α -amino acid group of K residues. Although UBLs share a similar fold, their functions and properties differ from Ub. Typically, only a small fraction of a protein competent for SUMOylation is usually SUMOylated, while proteases may execute de-conjugation reactions, including those of the posttranslationally formed isopeptide bonds found in mono-, multi- and polyUb chains or the very similar small SUMOs (Yates et al. 2016b) and conjugates (Schmaler and Dubiel 2010).

In plants as well as in other eukaryotes, the number of E3 ligases for NEDD8 and of NEDD8-modified proteins is much smaller when compared to Ub-modified ones (Hakenjos et al. 2013). Little is known about the function or conjugation of UFM1, MUB and HUB1 in plants. While knowledge of UFM1 is completely missing in plants, all E1, E2 and E3 enzymes for UFM1 conjugation have already been characterized in mammals (Daniel and Liebau 2014). MUB, a UBL present in animals, fungi and plants, is anchored to the membrane owing to the presence of a prenylation signal at its C-terminus instead of the usual di-glycine motif (Nagels Durand et al. 2016). In yeast and mammals, HUB1 functions non-covalently and no E1-E2-E3 cascade has been identified.

1.2.4 N-End Rule Pathway

The N-end rule relates the *in vivo* half-life of a protein to the nature of its N-terminal (Nt)-residue, which, alongside other requisite features (an unstructured, exposed N-terminus and accessible downstream K), forms a degradation signal called the “N-degron”. Proteins are produced with a methionine (Met; or formylmethionine, fMet) at their Nt (Xu et al. 2015; Frottin et al. 2006; Linster et al. 2015). In most proteins, Nt-Met is cotranslationally cleaved by METHIONINE AMINO-PEPTIDASES (MetAPs), exposing new Nt-residues. The N-end rule pathway has been co-opted to the UPS, targeting proteins for destruction by the UPS through conjugation of a polyUb chain (Zhang et al. 2017a; Gibbs et al. 2011, 2014; 2016; Gibbs 2015). N-degrons are typically conditional, exposed and recognized by Ub-E3 ligases (N-recognins) only under certain conditions.

We know two divisions of the N-end rule pathway: (1) the arginylation (Arg/) N-end rule, which recognizes substrates with unmodified basic or hydrophobic residues and (2) the acetylation (Ac/) N-end rule, which targets proteins bearing certain Nt-acetylated residues (Zhang et al. 2017a; Holman et al. 2009; Vicente et al. 2017). The confirmed “N-recognins” of the Arg/N-end rule: PROTEOLYSIS1 (PRT1) and PRT6 bind to substrates bearing aromatic or basic Nt-residues, respectively (Graciet et al. 2009; Garzon et al. 2007). During protein synthesis, the α -amino group of Nt-residues can be cotranslationally acetylated by ribosome-associated Nt-acetyltransferases (NATs) (Varland et al. 2015). This acetylation occurs either directly on Nt-Met or the 2nd residue after Met-removal by MetAP. Three NATs (NATA, B and C) catalyse the majority of these modifications, with each having distinct substrate specificities. Posttranslational Nt-acetylation also probably occurs.

NAT loss-of-function mutations cause growth defects and reduced photosynthetic efficiency (Gibbs 2015). The NatB loss-of-function mutant *tcu2* shows that Nt-acetylation regulates flowering time and leaf, inflorescence, flower, fruit and embryonic development. Furthermore, recent evidence shows that the N-end rule pathway is linked to meristematic activity in the shoot apical meristem in *Arabidopsis*. The molecular mechanism relates to the degradation of the MicroProtein LITTLE ZIPPER2 (ZPR2) which is degraded by the oxygen-dependent N-degron pathway and thus is stabilized at low O₂ levels (Weits et al. 2019). Key to the hypoxia-mediated regulation are the ERF-VII transcription factors (White et al. 2017). Similar to the aforementioned ZPR2 in the shoot meristem, the O₂-dependent N-end rule pathway degrades the ERF-VII factors which are thus stabilized at low O₂ (Gibbs et al. 2011). As explained below, limited proteolysis depends on a cohort of proteases (Liu et al. 2020; Tornkvist et al. 2019; Liu and Moschou 2018), which may also generate variation in protein turnover by exposing new Nt that can undergo various modifications or amend activities and functions by removing regulatory domains. An example is the BIG BROTHER protein described in Sect. 4.

1.3 Limited Proteolysis

Limited proteolysis is executed by endoproteases which cleave proteins in internal sites, while exoproteases (amino- or carboxypeptidases) trim protein ends (Willems et al. 2017). Proteases enzymatically hydrolyse peptide bonds, resulting in a widespread, irreversible posttranslational modification of the protein's structure and biological function (Fig. 1c). *Arabidopsis* and rice (*Oryza sativa* sp.) have close to 800 putative proteases, classified into clans and families. Proteases cleave peptide bonds by polarizing a normally unreactive carbonyl group of the substrate. The carbonyl oxygen is stabilized in an oxyanion hole, making the carbon atom more vulnerable to attack by an activated nucleophile. Proteases are classified by catalytic type and, at present, these are grouped into those in which the activated nucleophile

is a side-chain of an amino acid on the protease (“protein nucleophiles”) and those in which the nucleophile is an activated water molecule (“water nucleophiles”).

Protein nucleophiles can be serine-, threonine- or cysteine-type, while water nucleophiles can either be amino acids sidechains (aspartates or glutamates) or by metal ions bound by sidechains. The corresponding catalytic types of water nucleophiles are known as aspartyl-, glutamyl- or metallopeptidases. Furthermore, some proteases are orphan as their catalytic type remains either unknown or does not fall within any known category. Proteins not annotated as proteases may execute proteolysis. For example, in humans, the enzyme O-linked β -N-acetylglucosamine transferase (OGT) that has a canonical function in serine and threonine glycosylation also executes proteolytic maturation of the cell-cycle regulator host cell factor-1 (HCF-1) (Lazarus et al. 2013). Besides, not all proteases cleave proteins. For example, the *Arabidopsis* γ -glutamyl-transferase 1 and 2 (GGT1 and GGT2) hydrolyse the tripeptide glutathione, some members of the M20 protease family hydrolyse auxin-amino acid conjugates (Bartel and Fink 1995; Davies et al. 1999; Martin et al. 2007), while some carboxypeptidases show acyltransferase activity (sinapoyl-Glc accumulator 1 and 2) (Fraser et al. 2007). Recent evidence suggests that in animals the vast majority of deubiquitinases (i.e. proteases removing Ub from proteins) display isopeptidase and esterase activity (De Cesare et al. 2021). This finding is important, as many esterases could potentially also cleave proteins. We expect that this list of proteases that cleave other than peptide bonds will significantly expand soon.

Among other processes, diverse inhibitors regulate proteases, keep them inactive and, in many cases, a single protease may have more than one inhibitor, while the inhibitors may target more than one protease (Kumar et al. 2015). For example, the *Arabidopsis* inhibitors Serpin1 and disulfide isomerase-5 both interact with and inhibit the cysteine protease RESPONSIVE TO DESICCATION-21 (RD21) (Lampf et al. 2010). The interaction with disulphide isomerase-5 keeps RD21 inactive and it also functions as a chaperone, escorting RD21 en route from the endoplasmatic reticulum via the Golgi to vacuoles (Ondzighi et al. 2008). The acidic vacuolar environment activates RD21, which then contributes to protein degradation in senescing leaves (Otegui et al. 2005; van der Hoorn and Kaiser 2012). Such complex relationships between proteases and inhibitors create complicated proteolytic networks. This is further perplexed by the different inhibitory types: suicidal or reversible.

1.4 The Interplay Between Proteolytic Pathways: The Case of Autophagy-Limited Proteolysis

The best example of the interplay between limited proteolysis and autophagy is ATG8 cleavage by the cysteine protease ATG4. ATG8 is a small Ub-like protein, essential for the elongation and closure of the autophagosomal membrane (Yamasaki

et al. 2020). Upon induction of autophagy, ATG8 undergoes limited proteolysis followed by reversible lipidation. The C-terminus of ATG8 is cleaved by the protease ATG4 and the nascent C-terminal glycine is conjugated with a lipid phosphatidylethanolamine (PE) moiety via an amide bond, anchoring ATG8 in an autophagosomal membrane. Intriguingly, ATG8 is removed from the membrane of a mature autophagosome by the same ATG4 protease, which can hydrolyse the amide bond between ATG8 and PE. This ATG4 dual function is crucial for the formation and elongation of the autophagic membrane, proper localization and recycling of ATG8, and finally removal of ATG8 from the mature autophagosomes, which seems necessary for their subsequent fusion with the vacuole (Pérez-Pérez et al. 2021).

ATG4 is the only protease identified among known ATG proteins and is called also “autophagin” (Ding et al. 2018; Rawlings 2013; Vizovišek et al. 2018; Minina et al. 2017a). The balance between proteolytic and delipidating ATG4 activities defines ATG8-PE amount present in the cell. Unlike yeast, most of animal and plant genomes contain several orthologs of ATG4 and ATG8 genes, indicating potential sub- and/or neofunctionalization. To our knowledge, until very recently, only mammalian ATG8 orthologs had been shown to have different roles in certain types of selective autophagy (Lystad et al. 2014). While plant ATG8 orthologs have not yet been sufficiently characterized, assessment of the specificity of two autophagin orthologs towards nine ATG8s in *Arabidopsis* has revealed only slight selectivity but differences in their proteolytic activity and sensitivity (Seo et al. 2016). Moreover, recent advances have shown the involvement of different isoforms of ATG8 specifically in the regulation of selective autophagy pathways in plants (Zess et al. 2019).

Calpain-dependent cleavage of ATG5 (Yousefi et al. 2006) or caspase-dependent cleavage of ATG6 (Wirawan et al. 2010) in mammals yields protein fragments that cannot sustain autophagosomes formation, but instead activate apoptotic cell death in animals. N-terminal domain of the catalytically inactive protease ATG4D leads simultaneously to activation of the ATG4D and to release of the cytotoxic C-terminal domain of the protein (Betin and Lane 2009). Thus, limited proteolysis of these proteins switches cellular stress response from survival to death. Likewise, in *Arabidopsis* cleavage of the plant Bcl2 associated athanogene 6 (BAG6) by the aspartyl protease APCB1 induces autophagosome formation and restricts necrotic lesions spreading induced by the necrotrophic fungus *Botrytis cinerea* (Li et al. 2016b). Another example of an interplay between limited proteolysis and autophagy in plants is provided in Sect. 2.2.

2 Proteolysis in Reproductive Development and Embryogenesis

2.1 Reproductive Development

Flowers are the reproductive plant organs. Reproductive development is characterized by major metabolic changes. Through meiosis and mitosis, the male and female gametophytes also called the pollen grain and the embryo sac, respectively, are generated from anther tissue and ovules. Furthermore, a very important process for successful reproductive development is pollen germination followed by tube growth. This process is relatively fast and dynamic, characterized by vesicular trafficking and cytoskeletal changes, as well as highly active metabolism (Cameron and Geitmann 2018). These changes necessitate the use of proteolytic systems to remodel the proteome and thus it is hardly surprising that limited and digestive proteolysis regulate reproductive development.

The cysteine protease separase (known also as “Extra Spindle Poles”, ESP) executes the release of sister chromatid cohesion during meiosis and mitosis (Yang et al. 2009). ESP-dependent proteolytic cleavage of the α -kleisin subunit of the cohesin complex that holds sister chromatids together at the metaphase-to-anaphase transition is essential for the proper segregation of chromosomes (Minina et al. 2017b; Cromer et al. 2019). In *Arabidopsis*, meiotic-specific ESP RNA interference blocked cohesin removal from chromosomes and resulted in the presence of a mixture of fragmented chromosomes and intact bivalents leading to alterations in nonhomologous centromere association as well as disruption of the radial microtubule system after telophase II. Moreover, ESP RNA interference affects the proper establishment of nuclear-cytoplasmic domains, resulting in the formation of multinucleate microspores. Likewise, two *Arabidopsis* putative glycosylphosphatidylinositol (GPI)-anchored aspartic protease genes, A36 and A39, which are highly expressed in pollen and pollen tubes, play a role in reproductive development (Gao et al. 2017a, b). *a36 a39* mutants show precocious cell death of pollen and are female gametophytic defected. GFP-A36 and A39 localize at the plasma membrane and cytoplasmic puncta, colocalizing with the GPI-anchored protein COBRA-LIKE10 which plays a role in the cell wall structure and pollen tube guidance (Li et al. 2013). Hence, in *a36 a39*, the abundance of highly methyl-esterified homogalacturonans and xyloglucans is significantly increased in the apical pollen tube wall.

Aspartic proteases also play vital roles in tapetum degeneration timing, which is crucial in sexual reproduction (Olsson et al. 2019; Yang et al. 2012; Cecchetti et al. 2008). In *Arabidopsis* and rice, the aspartic proteases AtUNDEAD and OsAP25/OsAP37, respectively, modulate tapetal PCD timing; their absence leads to pollen abortion (Phan et al. 2011). In rice, S5 participates in indica-japonica hybrid fertility and could stimulate ER stress, giving rise to PCD in the embryo sac (Chen et al. 2020; Yang et al. 2012). OsAP65 is essential for pollen germination and tube growth (Huang et al. 2013). In *Arabidopsis*, loss of function of the ER-localized

PROMOTION OF CELL SURVIVAL1 (PCS1) aspartic protease causes gametophytic degeneration (Ge et al. 2005). Therefore, plant aspartic proteases may be implicated in the restriction of PCD in plant reproduction, although the underlying mechanism is not clear.

The MMS21 (HPY2) is a SUMO E3 ligase conserved in eukaryotes and required for DNA repair and chromosome integrity maintenance. In *Arabidopsis*, MMS21 loss-of-function causes defective meristems, dwarf phenotypes and gametophytic defects (Liu et al. 2014). SUMO E3 ligase MMS21/HPY2 represses the transition from the mitotic cycle into the endocycle in *Arabidopsis* (Ishida et al. 2012). The *hpy2-1* mutant survives for only a few weeks under normal growth, but a few seedlings eventually form shoots that show fasciation and defects in phyllotaxis. Their root meristems contain abnormally enlarged cells and a higher proportion of cells in the endocycle. These endocyclic cells also contain higher DNA content (reaching 64C and 128C) and much larger nuclei compared to wild-type. Mutants of MMS21/HPY2 that survive through the reproductive stage display severe fertility defects exemplified by a much-reduced seed set and increased rate of seed abortion (Ishida et al. 2012; Liu et al. 2014). Reciprocal pollination experiments suggested that most of the reduced fertility of *hpy2* mutants is likely due to pollen defects, although some defects in female gametophyte may also contribute to sterility but to a lesser extent. These and other experiments also suggested that *hpy2* pollen tube growth was defective even in wild-type pistils. These results are consistent with MMS21/HPY2 expression in anther and pollen. Further characterization revealed that *mms21/hpy2* anthers have morphological defects, are generally variable in size and shape and produce fewer and nonviable pollen grains compared with wild-type (Liu et al. 2014). Collectively, these results suggest that MMS21/HPY2 is required for male gametophyte development.

Regarding flowering, the SUMO ligase (*siz1*) and SUMO protease (*esd4*) mutations reduce the floral repressor *FLOWERING LOCUS C* (FLC) mRNA abundance and consequently enhance SOC1 expression which promotes flowering. Both mutants show compromised salicylate (SA) signalling; high SA levels associate with lower mRNA levels and accelerated flowering (Jin et al. 2008). Besides, SIZ1 may also activate FLC expression through an SA-independent pathway that requires the flowering time gene FLD (Jin et al. 2008). On the other hand, the FLC protein interacts with SIZ1, reducing the SUMOylation of FLC *in vitro* (Son et al. 2013). Furthermore, SIZ1 functions did not depend on SA levels because expressing *nahG* in *siz1* did not restore ovule viability, suggesting that SIZ1 plays a direct role in ovule development. Reciprocal pollination and pollen viability analyses revealed that *siz1* pollen is normal (Ling et al. 2012). On the other hand, *siz1* female gametophyte could not support full fertilization of wild-type pollen and scanning electron microscopy revealed that pollen grains germinate and pollen tubes migrate through the style of *siz1* plants but fail in the final stage of pollen tube guidance to reach the micropylar opening and hence fail to enter the embryo sac. Collectively, these findings suggest that SIZ1 is required for normal female gametophyte development. In the aforementioned cases, however, the role of these proteases in mechanistic terms is unclear.

Pollen tube growth is considered a costly and vigorous process because the cell needs to provide large amounts of the cell wall and membrane components, newly synthesized proteins and energy to fulfil growth demands. As described above, *atg6* causes male sterility due to the lack of pollen germination, but knockdown of this gene leads to morphologically normal pollen with decreased germination rate in comparison with wild-type plants (Fujiki et al. 2007; Harrison-Lowe and Olsen 2008). In rice *Osatg7* and *Osatg9* mutants showed complete sporophytic male sterility and decreased anther dehiscence under normal growth conditions, indicating that autophagy is crucial in reproductive development (Kurusu et al. 2014). Furthermore, pollen of these mutants appeared premature due to defects in anther during maturation, while heterozygous plants have normal pollen. Besides, the pollination of the heterozygous plants with wild-type resulted in normal fertility. These findings suggest that the cause of immature pollen phenotype displayed by autophagy-defective mutants depends on defects in various organs or parental tissue. In accordance, analyses of *Arabidopsis* and maize (*Zea mays*) plants harbouring mutations in the autophagy genes (*atg*) indicated that autophagy contributes to nitrogen remobilization from vegetative to reproductive tissues, including seeds (Have et al. 2017).

2.2 Embryogenesis

In this section, we focus on seed-carrying plants. In a plant seed, the embryo lies dormant surrounded by nutritive endosperm while awaiting suitable conditions to germinate. A hydrophobic cuticle around the embryo protects it from water loss during the early days of growth. Seeds carry large amounts of seed storage proteins, which serve as the primary source of nitrogen for the growing seedling during germination. In developing dicot seeds, the most abundantly expressed storage proteins are members of the 2S albumin and the 7S and 11S globulin protein families. Precursor polypeptides of these storage protein classes are synthesized at the ER, and the mature (processed) polypeptides of all of these three protein classes accumulate inside specialized vacuoles, called protein storage vacuoles (PSVs) (Delgadillo et al. 2020).

In the developing seed, a bidirectional molecular dialogue between embryo and endosperm safeguards cuticle integrity before germination and involves a limited proteolytic pathway (Doll et al. 2020). In this pathway, the ABNORMAL LEAF SHAPE1 subtilase produces the TWISTED SEED1 (TWS1), a peptide that acts as a GASSHO ligand and is recognized by the GASSHO receptor-like kinases. Cuticle surveillance depends on the action of the subtilase, which, unlike the TWS1 precursor and the GASSHO receptors, is not produced in the embryo but the neighbouring endosperm. Active TWS1 precursor mediates the GASSHO-dependent cuticle reinforcement in the embryo.

Autophagic cell death is considered one of the major programmed cell death (PCD) types in eukaryotes (Bozhkov and Jansson 2007). The most characteristic

example of autophagic PCD is the embryonic suspensor rupture. This organ is an early embryonic structure that links the embryo to the endosperm and its role is to mediate the transportation of nutrients and signalling molecules as well as to push the embryo through the endosperm (Peng and Sun 2018). In Norway spruce (*Picea abies*) the cysteine protease metacaspase type II (mcII-Pa) mediates lytic vacuole formation, thereby promoting suspensor death. *mcII-Pa* knockdown results in fewer autophagosomes in suspensor cells, however, the opposite is invalid, indicating that mcII-Pa activates autophagy and not vice versa (Minina et al. 2014; Reza et al. 2018). Moreover, ATG5 and ATG6 gene downregulation disrupts suspensor differentiation leading to alternative necrotic death and arrest of embryonic development. Yet, the molecular mechanism by which *mcII-Pa* modulates vacuole formation is unknown.

3 Proteolysis in Hormonal Regulation

Unlike hormonal regulation in animals, plant hormonal pathways depend on breaking points, i.e. proteins, with the ability to restrict a whole pathway. In this section, we provide examples of limited and digestive proteolysis and their effect on the regulation of development. Noteworthy, we present only a subset of direct functions that have been recently described as key regulators of hormonal signalling. The UPS of key regulatory proteins in hormonal pathways has been demonstrated or is at least likely, for all of the phytohormone response pathways.

3.1 Examples of Digestive Proteolysis in Hormonal Regulation

Here we provide selected examples of digestive proteolysis in hormonal regulation. More detailed discussion on the roles of UPS in hormonal pathways can be found in Kelley and Estelle (2012); Lopez-Obando et al. (2015) and autophagy in hormonal regulation in Gou et al. (2019); Liao and Bassham (2020).

3.1.1 Gibberellins

Plant embryos survive for a long time in a developmentally arrested state as dry seeds (i.e. dormancy), a state rapidly reversed during germination. The plant hormone gibberellin A (GA) promotes germination and dormancy loss when environmental conditions are favourable for germination and growth. GA stimulates plant growth and development by targeting the DELLA proteins for proteolytic degradation by the UPS. DELLA proteins are the key repressors of almost all GA responses. There are five DELLA proteins in *Arabidopsis*, the GA INSENSITIVE (GAI),

REPRESSOR OF *gal-3* (RGA), RGA-LIKE 1 (RGL1) and RGL2 (Van De Velde et al. 2017; Salanenka et al. 2018).

A good example of GA functions is the growth modulation in different light conditions. Light promotes plant photomorphogenesis, giving rise to open and expanded cotyledons, and short hypocotyls in *Arabidopsis* seedlings. In the dark, seedlings undergo de-etiolation (Lyu et al. 2019), characterized by closed cotyledons and elongated hypocotyls (Lyu et al. 2019). A subset of basic helix-loop-helix (bHLH) transcription factors, known as phytochrome-interacting factors (PIFs), has a key role in etiolation and light-regulated plant development. Upon illumination, the photoactivated phytochromes trigger PIFs' rapid phosphorylation and subsequently UPS-mediated degradation, leading to transcriptional changes that promote photomorphogenesis. DELLAs promote PIF degradation through UPS. When GA is present, GA receptor *GID1* binds to DELLAs to form *GID1*-GA-DELLA complex, which then triggers DELLA proteins degradation by the UPS. DELLAs have a conserved DELLA domain (motif Asp-Glu-Leu-Leu-Ala) at the Nt, essential for GA-triggered protein degradation (Li et al. 2016a).

SUMO regulates GA signalling, for example through *SLEEPY1* (*SLY1*) SUMOylation by *SIZ1* (Mazur et al. 2019). *SLY1* is an F-box protein component of an SCF complex (*SCF^{SLY1}*) that mediates the interaction of *GID1* with DELLA proteins in response to GA, thereby facilitating DELLA degradation (Wang et al. 2009). *SIZ1* SUMOylates *SLY1*, this increases *SLY1* stability and interaction with DELLA proteins, promoting growth due to enhanced DELLA degradation (Kim et al. 2015). Interestingly, GA induces *SIZ1* expression and stimulates *SLY1* SUMOylation. These data propose that GA stimulates growth by inducing *SIZ1* expression, which in turn leads to *SLY1* sumoylation, stabilization and interaction with DELLA proteins. *SLY1* interaction with DELLA leads to subsequent DELLA degradation. This model implicates SUMO as a positive regulator of GA-mediated plant development.

3.1.2 Jasmonates

Jasmonic acid (JA) together with its precursors and derivatives, referred to as jasmonates (JAs), regulate – among other processes – the response to wounding, by inducing specialized metabolism (Nagels Durand et al. 2016). JAs further contribute to plant plasticity by regulating responses to abiotic stresses, thereby adjusting growth and productivity under adverse conditions. Finally, JAs are also important in the regulation of responses to light and development (Zhou et al. 2019).

The core JA-signalling module comprises (1) *MYC2*, a key transcription factor regulating expression of JA-responsive genes, (2) Jasmonate ZIM-domain (JAZ) proteins, repressors that inhibit *MYC2* activity in the absence of the hormone and (3) *CORONATINE INSENSITIVE 1* (*COI1*) that acts as the JA receptor *in vivo* and, in response to JA-Ile, targets the JAZ repressors for proteolytic degradation. The co-repressors Novel Interactor of JAZ (*NINJA*) and *TOPLESS* (*TPL*) mediate the repressing effect of JAZ proteins (Santner and Estelle 2007). In the absence of JAs,

members of the JAZ protein family repress expression of JA-responsive genes by inhibiting MYC2 activity. Upon treatment with the hormone, JAZ proteins interact with COI and are degraded by UPS, thereby allowing JA-responsive genes activation. In the absence of JAs, members of the JAZ protein family repress expression of JA-responsive genes by inhibiting MYC2 activity. Upon treatment with the hormone, JAZ proteins are degraded by the UPS, thereby allowing transcriptional activation of JA-responsive genes. This degradation depends on the direct interaction between JAZ and COI1 (Nagels Durand et al. 2016). JAZ proteins contain three conserved domains: the zinc-fingers expressed in the inflorescence meristem (ZIM) domain, a region of weak homology at the N-terminus and the C-terminal Jas domain which is most strongly conserved. The Jas domain is essential for JAZ stability because it constitutes the interaction platform between JAZ and COI1 upon hormone treatment. The Jas domain of JAZ repressors is required for both the formation of the COI1-JA-Ile-JAZ co-receptor complex and the interaction with the JID domain of MYC TFs.

3.2 Examples of Limited Proteolysis in Hormonal Regulation

3.2.1 Auxin

The plant hormone indole-3-acetic acid (IAA or auxin) regulates many developmental processes and stress responses, acting as permissive or restrictive signal depending on concentrations of active auxin or tissue/cell context. Auxin promotes the recognition of the transcriptional repressors Aux/IAAs “degron” domain II (DII) by the E3 Ub-ligase proteolytic complex Skp1–Cullin1–F-box (SCF)^{TIR1/AFB}, leading to their degradation in the nucleus. In the apical hook, a parallel cytoplasmic pathway commences at the cell membrane and involves TMK1 (Cao et al. 2019). TMK proteins contain an intracellular kinase domain, a single transmembrane pass and an extracellular domain with two leucine-rich repeats separated by a non-LRR region. *tmk1* loss-of-function mutants have disrupted apical hook development. TMK1 shows a transient cytosolic and nuclear distribution at the concave side of the apical hook. The redistribution depends on the cleavage of TMK1 by an unknown pathway that coincides with local auxin maxima, releasing a fragment containing the intracellular kinase domain, dubbed as TMK1-C (Cao et al. 2019).

In strict contrast to the Skp1–Cullin1–F-box (SCF)^{TIR1/AFB} digestive pathway, TMK1-C stabilizes IAA repressors (Cao et al. 2019). The stabilization step involves the translocation of TMK1-C from the plasma membrane to the cytosol and nucleus where it interacts with and phosphorylates IAA32/34. The two proteins lack the DII and thus are not degraded by SCF^{TIR1/AFB}. In the *tmk1*, IAA32/34 proteins decreased, and auxin could not induce their accumulation. However, it is unclear what determines the stability of IAA32/34 and these proteins could contain cryptic degrons or are degraded by non-UPS pathways such as exoproteases.

Another example of a limited proteolytic pathway regulating auxin is the aforementioned *Arabidopsis* ESP (see Sect. 3.1). ESP regulates auxin gradient in the root meristem by adjusting PINFORMED (PINs) proteins which give rise to directional polar auxin transport and contribute to auxin levels regulation (Liu et al. 2017, 2020; Moschou et al. 2016). This regulation is accomplished through a collaboration between ESP and microtubule-binding kinesins (Kin7.3-clade), affecting microtubule stability and delivery of PINs to polar plasma membrane domains. There, PINs transport auxin contributing to a certain distribution. Although microtubules are important in the regulation of PIN polarity, the molecular mechanism by which ESP executes the polarization of PINs is unknown.

3.2.2 Ethylene

Ethylene is a volatile plant hormone involved in many plant processes such as ripening, ageing and senescence, and is produced in all plant organs. Ethylene is perceived at the ER membrane (Chao et al. 1997). When ethylene is absent, specific ethylene receptors form complexes with the protein kinase CONSTITUTIVE TRIPLE RESPONSE-1 (CTR1) and the integral membrane protein ethylene insensitive 2 (EIN2), a process which leads to EIN2 phosphorylation by CTR1 (Hua and Meyerowitz 1998).

EIN2 is a general regulator of the ethylene signalling pathway. When ethylene is present, it acts as an inverse agonist by inhibiting its receptors. EIN2 is, then, released, dephosphorylated and cleaved releasing a fragment with two functions. On the one hand, the EIN2 C-terminal part is translocated to the nucleus, where it stabilizes the EIN3 transcription factor via the EIN2 nuclear-associated protein 1 (ENAP1), resulting in the activation of ethylene response genes (Wen et al. 2012). On the other hand, the EIN2-C is bound, occasionally, to EBF1 and EBF2 mRNAs, where this complex is associated with processing bodies in the cytoplasm, leading to degradation. Degradation of EBF1 and EBF2 releases, among others, EIN3 and results thus in more ethylene signalling. Interestingly, the protease executing EIN2 cleavage is unknown.

3.3 Peptidic Hormone-Like Molecules

Small posttranslationally modified peptides are signalling molecules involved in many aspects of plant growth and development (Stührwohldt et al. 2020). One of the posttranslational modifications that peptides undergo is proteolytic cleavage. As exemplified in Sect. 3.2, subtilisins (SBTs) are amongst the most probable candidates for diffusible peptide production. SBTs are usually produced as inactive proteins, activated upon removal of their inhibitory leading peptides through autoprocessing. Once auto-processed, they cleave substrates in the apoplast (Vartapetian et al. 2016).

The discovery of SBT substrates and their functional studies have been obscured by extensive redundancies within the large SBT family (56 members in *Arabidopsis*; (Schardon et al. 2016). A notable exception of SBTs' "redundancy rule" is SBT5.2 that modulates the density of stomata in high levels of CO₂, by producing the mature peptide EPIDERMAL PATTERNING FACTOR 2 (EPF2). In the apoplast, EPF2 inhibits epidermal cells from adopting a meristemoid mother cell fate (MMC; (Engineer et al. 2014). By combining expression profiling with proteomic studies, Engineer et al. identified SBT5.2 as the potential protease cleaving EPF2. EPF2 cleavage by SBT5.2 was confirmed *in vitro* and the double loss-of-function mutant *epf2sbt5.2* had no additive phenotypes compared with the single mutants, suggesting that EPF2 and SBT5.2 act together. EPF2 overexpression in the *sbt5.2* background reduced stomata to a lesser extent than in the wild-type, indicating SBT5.2 involvement in EPF2-peptide maturation.

Peptides can mature in a stepwise manner in different cell compartments. The peptides CLEL6 and CLEL9 (also known as GOLVEN 1 and 2) control gravitropic responses of the shoot and the root by modulating auxin distribution (Stührwohldt et al. 2020). Several proteolytic processing events located in consecutive compartments of the secretory pathway are required for maturing CLEL peptides. Using an inhibitor-based approach for loss-of-function analysis, targeting protease function at the level of enzyme activity it was shown that the stepwise maturation of CLEL peptides is mediated by SBTs. Following the cleavage of the signal peptide upon entry into the ER, the CLEL6 and 9 precursors are processed at two sites by SBT6.1. Cleavage by SBT6.1 in the *cis*-Golgi allows for the continued passage of the partially processed (pre-activated) precursors through the secretory pathway and is thus a prerequisite for subsequent posttranslational modifications including tyrosine sulfation and proline hydroxylation within the Golgi, and proteolytic maturation after exiting the Golgi (Stührwohldt et al. 2020). The activation of CLEL6 and CLEL9 by SBTs in the *trans*-Golgi network or other post-Golgi compartments depends on the Nt aspartate of the mature peptides.

As mentioned earlier, extensive redundancies within SBTs (or other protease families) make them refractory to functional studies. To overcome this limitation, Schardon et al. expressed heterologous SBT inhibitors from *Phytophthora infestans*, called extracellular proteinase inhibitors (EPIs; EPI1 and EPI10), under specific promoters active during floral organ abscission. EPI expression blocked the production of the diffusible, (IDA) mature form (mIDA), which controls floral abscission in *Arabidopsis* (petals, sepals and stamens), and a similar pathway functions in root cap sloughing (Shi et al. 2018). The SBT4.12, SBT4.13 and SBT5.2, expressed during abscission, could cleave IDA *in vitro*. Although these data are insufficient to conclude how many SBTs are involved in the abscission, they provide evidence for SBTs involvement in this process.

4 Proteolysis in Leaf Development and Endoreduplication

During leaf development, the balance between cell proliferation and differentiation is highly coordinated even before the new leaf emerges and grows. Endoreplication permits multiple rounds of DNA replication without subsequent cell division (cytokinesis), leading to the successive doubling of the nuclear DNA content of the cell (Dissmeyer et al. 2009). The transition from a mitotic cell cycle into an endocycle is often associated with the switch of meristematic cells from division to expansion and differentiation. Proteolysis ensures the unidirectional progression of the cell cycle by causing irreversible changes in key factors that direct this process. The cyclin-dependent kinase inhibitor KRP2 is considered a crucial component to whether the cell will undergo endoreplication through the regulation of mitotic Cyclin-Dependent Kinase (CDK)A;1 complex (De Schutter et al. 2007). More specifically, KRP2 expression levels are regulated posttranslationally via proteolysis in a phosphorylation-dependent way and the abundance of this protein determines the inhibition of CDKA;1, thus, resulting in mitotic progression or endocycle entrance.

Previous studies showed that regulated proteolysis, especially UPS, is considered an important factor for leaf growth. Two E3 ligases, BIGBROTHER (BB) and HISTONE MONOUBIQUITINATION (HUB1, and its homolog HUB2) define cell proliferation phase length and rate in leaves by tagging with Ub the proteins that need to undergo degradation. More specifically, HUB1 can mono-ubiquitinate histone H2B *in vitro* and the corresponding knockdown mutants showing increased cell cycle duration in young leaves. That led to reduced rosette biomass, pale leaves and changes in leaf shape (Fleury et al. 2007). Regarding the BB protein, changes in expression levels lead to different organ sizes and BB abundance correlates with cell proliferation (Cattaneo and Hardtke 2017). The *Arabidopsis* Ub-activated protease DA1 limits the duration of cell proliferation during organ growth. DA1 is activated by BB and another RING E3 ligase and the DA2. The BB and DA2 are cleaved by DA1 and this cleavage leads to their destabilization (Dong et al. 2017). The cleavage of BB leads to destabilization via the N-end rule pathway-related protein PRT1. DA1 protease also cleaves the de-ubiquitinase UBP15 and the transcription factors TEOSINTE BRANCHED 1/CYCLOIDEA/PCF 15 (TCP15) and TCP22 which repress endoreduplication and promote cell proliferation. Furthermore, another example of cell cycle regulation through proteolysis during leaf development is the Anaphase-Promoting Complex/Cyclosome (APC/C) which is a Ub E3 ligase that targets mitotic cyclins for degradation by the UPS (Kondorosi et al. 2005). Eloy et al. overexpressed the subunit 10 of this complex and observed enhanced leaf size due to increased APC/C activity that led to higher cell division rates, while knockout mutant showed strong defects in female gametogenesis (Eloy et al. 2011).

Finally, another CDK activity repression pathway includes the SIAMESE(SIM) gene that encodes the founding member of the SIAMESE-RELATED family. While

normally, the trichomes of *Arabidopsis* undergo three to four endoreplication cycles, in *sim* mutants this does not occur due to endocycle repression, leading them to divide mitotically (De Veylder et al. 2011). Furthermore, the SIM homolog SMR1/LOSS OF GIANT CELLS FROM ORGANS (LGO) is upregulated in developing leaves and sepals, indicating a more general role in modulation of endoreplication transition during organ emergence (Roeder et al. 2012).

5 Proteolysis in Senescence

Senescence is the last developmental phase that eventually results to death of single cells, tissues or even the whole plant. This stage is highly regulated and accompanied by an increased rate of protein degradation. It can also function as a recycling process by which the nutrients accumulated in senescing tissues are remobilized to be used for the production of new vegetative, reproductive or storage organs. While the mRNA levels of most genes decline, those of “senescence-associated genes” (SAGs) increase during senescence. As protein degradation is a vital process for successful nutrient recycling, it is not surprising that many of the upregulated genes in senescing tissues encode proteases (Bhalerao et al. 2003).

Among the many classes of plant proteases, mostly serine and cysteine-dependent proteases associate with senescence across different species (Diaz and Martinez 2013). Serine proteases are the largest protease class in plants and evidence shows that they are the dominant proteases during senescence (van der Hoorn 2008). For example, two SBTs (P1 and P2) are activated during dark and nitrogen-starvation induced senescence in wheat and execute ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) degradation in developing grains (Roberts et al. 2011).

At least three cysteine proteases are induced in the vacuole during dark-induced senescence in wheat (Martínez et al. 2007). Another cysteine protease is the SAG12, a papain-like protease. However, the physiological role of SAG12 is not known; the loss-of-function *sag12* mutant neither fails to develop senescence-associated vacuoles nor shows any morphological phenotype. The Vacuolar Processing Enzyme (VPE) cysteine protease is responsible for vacuolar protein maturation and promotes cell death during stress and development (Nakaune et al. 2005). In *Arabidopsis*, the isoforms α VPE and γ VPE are upregulated in vegetative organs during senescence (Sanmartin et al. 2005). Finally, plant metacaspases are upregulated in *Arabidopsis* during senescence in leaves (MC6 and MC9) and flowers (MC3 and MC9), although their exact mechanistic function remains elusive (Breeze et al. 2011).

The class of aspartic proteases is also linked to senescence, although their role is less documented than cysteine protease. Proteomic studies in different species such as oilseed rape (*Brassica napus*) and rice showed high levels of aspartic proteases, throughout senescence (Desclos et al. 2009). The most characteristic example of aspartic protease role in plant senescence is CND41 in tobacco (Kato et al. 2004,

2005). This protease localizes in chloroplast and degrades Rubisco *in vitro* at physiological pH. Besides, silencing of CND41 resulted in Rubisco accumulation in older leaves and delayed senescence suggesting deficient remobilization machinery, while overexpression led to accelerated senescence and higher Rubisco degradation in senescent leaves.

Metalloproteases have a role in senescence but to a lesser extent. The most studied metalloprotease family in plants is the FtsH, which is a family of membrane-bound ATP-dependent proteases that contain a zinc-binding domain. Twelve genes are encoding FtsHs in the nuclear genome of *Arabidopsis*, and nine of them localize in chloroplasts while the others are found in the thylakoid membrane (Kato and Sakamoto 2010). Many FtsHs are upregulated during senescence in *Arabidopsis* (Guo et al. 2004). Furthermore, studies in *Brassica napus* senescence under nitrogen starvation led to the induction of the chloroplast-localized FtsH protease at the early stages of senescence while decreasing at the end, indicating a role in early protein degradation in chloroplasts (Desclos et al. 2009).

With regard to digestive proteolysis, in both plants and mammals, autophagy has direct effects in triggering and executing cellular senescence. As mentioned above, recycling of inefficient photosynthetic organs provides plants with nutrient supplies to progress their life cycle at the whole-plant level and sustains seed quality via efficient seed filling. More specifically, leaf senescence is a dynamic process that responds to source/sink demands of the plant regulated by stress responses, nutrients sensing and phytohormones (Buchanan-Wollaston 1997; Lim et al. 2003; Guiboileau et al. 2010). Autophagy plays an important role during senescence as defects in autophagy generally have almost normal development but are more sensitive in nitrogen and carbon starvation or progress to early senescence in low light conditions (Yoshimoto et al. 2014; Thompson et al. 2005; Chung et al. 2010). One exception is the loss-of-function *atg6* knockout mutant which shows defective pollen germination (Fujiki et al. 2007) and therefore there can be no homozygous mutants. On the other hand, in mammals, knockout of ATG genes may lead to embryo lethality while from a molecular point of view, elevated levels of autophagy activation may induce cell death, whereas decreased autophagy triggers cellular senescence (Kuma et al. 2005; Rajendran et al. 2019). Furthermore, loss-of-function *atg9* mutants in *Arabidopsis* showed early leaf chlorosis (degradation of chlorophyll) under nitrogen or carbon-starvation conditions (Zhuang et al. 2017).

6 Organellar Proteolysis and Development

6.1 Mitochondria

In mitochondria, protein turnover is regulated externally via anterograde/retrograde communications with the nucleus and internally via proteolysis and chaperones that act posttranslationally on the proteome. Mitochondrial protein turnover is controlled by ATP-dependent proteases involved in the degradation of misfolded proteins or

limited proteolytic cleavage of pre-sequences, resulting in protein maturation. ATP-dependent proteases have two conserved domains, an AAA+ domain which enables the protease to bind ATP and performs as a chaperone and a proteolytic domain with a Serine-K catalytic dyad (Lee and Suzuki 2008; Rigas et al. 2009). Among others, long-filament phenotype-1 (LON1), caseinolytic protease (CLPP) and filamentous temperature-sensitive H (FTSH) are the most dominant and conserved components of the proteolytic networks in eukaryotes (van Wijk 2015).

CLPP is an energy-dependent serine-type protease that plays a role in protein quality control. The CLPP protease has an active Serine-Histidine-Aspartate catalytic triad and is present widely amongst bacterial species as well as fungal, mammalian and plant mitochondria (Bhandari et al. 2018). In plants, knockouts of the single gene for CLP protease subunit, *clpp2*, did not result in growth or development defects, nuclear transcripts were unaffected, whereas mitochondrial genes encoding oxidative phosphorylation protein complexes were abundant (Petereit et al. 2020).

LON1 is a highly conserved mitochondrial protein family in eukaryotes acting as a chaperone facilitating the proper folding of newly synthesized or imported proteins in the mitochondria and as a protease removing protein aggregates (Li et al. 2017). Mitochondrial LON1 loss impairs oxidative phosphorylation complexes and tricarboxylic acid cycle (TCA) enzymes. Phenotypically, LON depletion in *Arabidopsis* restricts root growth and leads to deleterious, developmental phenotypes (Rigas et al. 2009). When LON1 is disrupted, oxidative phosphorylation proteins are less abundant, resulting in heat shock proteins and prohibitin accumulation in the mitochondria (Solheim et al. 2012).

FtSH proteases are membrane-bound metalloproteases family abundant in eubacteria, animals and plants. So far, we know four FtSH genes in *Arabidopsis* mitochondria; FtSH3 and FtSH10 are mitochondrial AAA proteases, while FtSH4 and FtSH11 function as mitochondrial inner membrane AAA proteases (Kato and Sakamoto 2010). More specifically, FtSH4 is involved through reactive oxygen species accumulation, in phytohormone signalling and transcriptional regulation (Zhang et al. 2017b). FtSH4 substrates include the inner membrane translocase Pam18-2 and mitochondrial pyruvate transporter 4 (MPC4) (Opalińska et al. 2017). In *Arabidopsis* FtSH4 loss of function results in abnormalities of rosette leaves when grown under short-day conditions, structural alterations in mitochondria, accompanied with increased reactive oxygen species levels, carbonylated mitochondrial proteins, reduced cardiolipin contents (Gibala et al. 2009), oxidative phosphorylation subunits alterations (Heidorn-Czarna et al. 2018) and Hsp70 and prohibitins accumulation (Gibala et al. 2009). Additionally, evidence shows that FtSH4 is involved in auxin homeostasis, regulating plant growth and development (Zhang et al. 2014), as well as the regulation of autophagic-PCD and senescence through SA homeostasis (Huang et al. 2018). Moreover, FtSH4 is essential for oxidative phosphorylation subunits biogenesis and, thus, biogenesis of mitochondria during germination (Heidorn-Czarna et al. 2018). Overall, though, recent studies show that mitochondrial AAA proteases, despite their importance in translation and

mitochondrial proteome surveillance, do not seem to have serious morphological modifications in plants grown under normal conditions (Kolodziejczak et al. 2018).

6.2 Chloroplasts

Nucleus-encoded proteins constitute a large part of the organellar proteome. These proteins are imported by multiprotein translocases (TOC) in chloroplast envelope membranes. Prokaryotic-type proteases control internal chloroplast protein turnover. So far, we know three procaryotic-like proteases modulating chloroplast protein turnover: FtsH, Deg and Clp. Overall, we so far know 20 chloroplast proteolytic types. Specifically, processing peptidases and energy-driven processive proteases are the major players in chloroplast proteome biogenesis, remodelling and maintenance.

6.2.1 The CHLORAD Pathway

The most well-studied example of proteolysis in chloroplasts is photosystem turnover regulation during excess light which can damage the photosynthetic mechanism. More specifically, photosystem II reversible phosphorylation and dephosphorylation lead to the E3-dependent ubiquitination and degradation of its D1 domain. This D1 domain has to be synthesized again *de novo* so that the photosystem II complex can perceive light and participate again in the photosynthetic pathway (Nath et al. 2013). On the other hand, targeted protein turnover at the chloroplast outer membrane is perhaps mediated by cytoplasmic proteolytic pathways. For example, the outer membrane CDC48 is an Omp85-type channel and SP2 is a cytosolic AAA+ chaperone that fulfils conductance and motor functions, respectively, in the retrotranslocation of target proteins from chloroplasts. This D1 domain has to be synthesized again *de novo* so that the photosystem II complex can perceive light and participate again in the photosynthetic pathway (Nath et al. 2013). CDC48 extricates proteins from the chloroplast membrane (they are integral membrane proteins). This process exposes outer membrane proteins to the UPS, of the substrates tagged by the chloroplast-localized E3 ligase SP1. In accordance, SP1, SP2 and CDC48 physically interact and form a complex at the chloroplast membrane. This proteolytic system is known as “chloroplast-associated protein degradation”, or CHLORAD (Ling et al. 2019).

Yet, we anticipate that many more proteins are CHLORAD components and possible links to chlorophagy (see below), a specialized type of autophagy targeting chloroplasts (van Wijk 2015; Izumi and Nakamura 2018; Ding et al. 2018; Tornkvist et al. 2019; Have et al. 2017). We should note, for example, that in mammalian cells, Ub-tagging largely acts as a trigger of autophagic removal of dysfunctional organelles (Marmor-Kollet et al. 2020). For example, during mitophagy (i.e. mitochondrial autophagy), dysfunctional mitochondria are tagged

by the E3 ligase Parkin, allowing for the autophagic removal of tagged mitochondria (MacVicar et al. 2019; Jin and Youle 2012). We should note, for instance, that in mammalian cells, Ub-tagging largely acts as a trigger of autophagic removal of dysfunctional organelles (Marmor-Kollet et al. 2020).

6.2.2 The Autophagy-Rubisco Degradation Pathway

Chloroplasts have specialized proteolytic machineries for protein degradation during senescence. Whereas stromal proteins decrease during the earlier stages of leaf senescence in wheat or barley (*Hordeum vulgare*), chloroplast numbers/cell decrease later (Kubínová et al. 2013). Therefore, stromal proteins can degrade without the breakdown of the chloroplast. Immuno-electron microscopy (EM) analysis of Rubisco degradation in senescing wheat (*Triticum aestivum*) leaves revealed the presence of cytosol-localized small vesicles (~1 µm) that contained Rubisco, but not thylakoid proteins (Izumi and Nakamura 2018). While stromal proteins decrease during the earlier stages of leaf senescence in wheat or barley (*Hordeum vulgare*), chloroplast numbers/cell decrease later (Kubínová et al. 2013). Therefore, stromal proteins appear to degrade either inside or outside the chloroplast without the breakdown of the entire chloroplast. These vesicles are frequently surrounded by autophagosome-like double membranes and were originally referred to as Rubisco-containing bodies (RCBs). In *Arabidopsis* and rice leaves expressing stroma-targeted green fluorescent protein (GFP) or GFP-labeled Rubisco showed that RCBs are not produced in the *atg5* or *atg7* mutants and that RCBs co-localized with the autophagosomal marker, GFP-ATG8. These observations reveal that RCBs are a type of autophagic body that delivers a portion of the stromal proteins into the vacuole (Otegui 2017).

ESCRT is part of an evolutionarily conserved system responsible for endosomal membrane remodelling. In *Arabidopsis*, the endosomal sorting complex required for transport (ESCRT)-III paralogs charged multivesicular body protein 1A (CHMP1A) and CHMP1B and delivered RCBs to the vacuole (Spitzer et al. 2015). In loss-of-function *chmp1a chmp1b* mutants, RCBs accumulated in the cytoplasm; therefore, CHMP1 proteins sort RCBs. How a portion of the stroma is separated as RCBs, and how RCBs are then recruited for autophagic transport remains unclear.

The RCB pathway is particularly active in sugar-starved, excised *Arabidopsis* leaves in darkness or the presence of photosynthesis inhibitors. The starchless mutants, phosphoglucomutase (*pgm*) and ADP-glucose pyrophosphorylase1 (*adg1*), lacking starch, showed enhanced RCBs production (Izumi et al. 2010). Moreover, starchless and *atg* double mutants have reduced growth and enhanced senescence compared to the respective single mutants. These findings indicate that the RCB pathway mediates nitrogen remobilization from older leaves that cannot acquire sufficient light due to the shading of developing leaves by upper tissues. Analyses of *Arabidopsis* and maize (*Zea mays*) plants with *atg* mutations showed that autophagy contributes to nitrogen remobilization from vegetative tissues to reproductive tissues and seeds (Li et al. 2015). However, such a role for autophagy

in rice plants was not evaluated, because autophagy-deficient rice plants exhibit male sterility due to impaired pollen maturation (Hanamata et al. 2019; Sera et al. 2019).

Some isolated vacuoles from the darkened leaves of wild-type plants contained chloroplasts that exhibited chlorophyll autofluorescence signals. These findings suggest that shrunken chloroplasts, which are produced through the active separation of their components in the RCB pathway, become the targets of autophagic transport as entire organelles, a process known as chlorophagy (Yamauchi et al. 2019). ATG8-interacting protein 1 (ATI1) and ATI2 were identified in a yeast two-hybrid screen as candidates that interact with the *Arabidopsis* ATG8 isoform, ATG8f (Wu et al. 2021; Michaeli et al. 2014). These proteins associate with plastids (in addition to the ER) as small vesicles of approximately 1 μm in diameter, which are referred to as ATI bodies. These delivery cargos differ from those of the RCBs that specifically contain a portion of stroma; however, the vacuolar transport of plastid-associated ATI bodies is an autophagy-dependent process, as ATI was not produced in *atg5* mutants (Michaeli et al. 2014). Therefore, ATI bodies represent a distinct form of autophagy vesicles that transport some stroma, thylakoid and envelope components into the vacuole. Plastid-associated ATI bodies are also observed inside the chloroplast, and ATI1 interacts with some thylakoid proteins *in vivo*. It is thus conceivable that plastid-associated ATI bodies form in chloroplasts and are then delivered into the vacuole via autophagosome-mediated transport, although how such bodies are evacuated from chloroplasts remains unclear.

6.2.3 The SAV Pathway

Senescing leaves of *Arabidopsis*, soybean (*Glycine max*) and tobacco (*Nicotiana tabacum*), and perhaps other plants, show small, lytic senescence-associated vacuoles (SAVs). SAVs are stained by the R-6502 dye, emitting strong fluorescence upon the hydrolytic activity of cysteine proteases (Martínez et al. 2008). SAVs form in the peripheral cytoplasmic region of mesophyll cells and are much smaller than the central vacuole, but with much greater lytic activity. SAVs contain SAG12, stromal proteins such as Rubisco and glutamine synthetase, but not thylakoid proteins such as LHCII and the photosystem II reaction centre D1 (Martínez et al. 2008). As *atg7* mutants had SAVs, they may be an autophagy-independent, extra-chloroplastic route for stromal protein degradation in senescing leaves. The trafficking pathway of stromal proteins into the SAVs, as well as their composition in proteases, remains uncertain.

6.2.4 The CV Pathway

Another type of organelle related to protein degradation is chloroplast vesiculation (CV), which depends on a protein produced by the CV gene. In *Arabidopsis*, CV-GFP expression under the dexamethasone-inducible promoter caused the formation of a type of chloroplast-derived vesicle showing a strong signal referred to as

CV-containing vesicles (CCV) (Kamranfar et al. 2018). CCVs are around 1 μm and contain stroma, envelope and thylakoid proteins, as shown by immunoblot analysis of some chloroplast proteins, co-immunoprecipitation assays and detection of fluorescently tagged microscopy of stroma markers. CCVs do not associate with GFP-ATG8a and SAVs, and the *atg5* mutation does not affect CCVs. Thus, CCVs are part of a vacuolar degradation process for chloroplasts that is independent of autophagy and SAVs.

6.2.5 “N-end rule” Pathway in Chloroplasts

Similar pathways to N-end rule may also function in organelles. Nuclear-encoded proteins comprise > 95% of the chloroplast proteome and are targeted to the plastid by an Nt-chloroplast transit peptide (cTP). Upon delivery to the chloroplast, the cTP is removed by the stromal processing peptidase (SPP) to expose new Nt-amino acids, which can then be further modulated by one of at least seven aminopeptidases (van Wijk 2015). SPP cleaves at a range of different sites, and single or multiple positions; this enzymatic promiscuity coupled with subsequent amino-peptidase activity may ensure the removal of unfavourable (potentially destabilizing) Nt-residues (Rowland et al. 2015). Furthermore, plastid-encoded proteins initiate with Nt-fMet and undergo cotranslational de-formylation followed by Nt-Met excision, both essential for normal plastid development (Gigliione et al. 2014). Interestingly Met-retention on chloroplast proteins associates with protein instability (Gigliione et al. 2003), whilst fMet can act as a destabilizing residue in bacteria, and possibly also chloroplasts (Piatkov et al. 2015). Cotranslational and posttranslational Nt-acetylation also occurs on chloroplastic proteins, which appears to enhance protein stability (Bienvenut et al. 2011); a nuclear-encoded chloroplast-targeted NAT probably catalyses this modification (Disch et al. 2006).

6.3 Peroxisomes

Peroxisomes are organelles, abundant in all eukaryotes and essential for development and stress responses. Some of their important cellular functions are fatty acid β -oxidation, photorespiration, ureide and polyamine metabolism, biosynthesis of plant hormones and reactive oxygen/nitrogen species production (Moschou et al. 2008; van Wijk 2015). Peroxisomes metabolize the auxin indole-3-butyric acid (IBA) into the active auxin indole-3-acetic acid (IAA) (reviewed in Strader and Bartel (2011)). Mutants with dysfunctional peroxisomes often display impaired IBA-to-IAA conversion (Strader and Bartel 2008) and dampened IBA responsiveness (Zolman et al. 2008).

Arabidopsis peroxisomes contain several proteases, e.g. LON2 (Lon protease 2), DEG15 (Degradation of periplasmic protein 15), SCPL20 (Serine carboxypeptidase-like protein 20), RDL1 (Response to drought21A-like 1) and PXM16 (Peroxisomal

M16 metalloprotease) (Pan and Hu 2018). LON2 facilitates sustained matrix protein import in mature peroxisomes and the degradation of matrix proteins during peroxisome remodelling (Adebesin et al. 2018). LON2 facilitates sustained matrix protein import in mature peroxisomes and the degradation of matrix proteins during peroxisome remodelling (Adebesin et al. 2018). In watermelon, DEG15 can be a processing peptidase as a dimer to cleave PTS2 from PTS2-containing proteins or a general protease in its monomeric form (Helm et al. 2007). SCPL20 is involved in β -oxidation and plant pathogen response (Quan et al. 2010, 2013), and RDL1 plays a role in β -oxidation, seed viability and stress response (Quan et al. 2013). The exact role of RDL1, SCLP20 and PXM16 remains to be elucidated.

Plant peroxisomal proteome varies to some extent in different developmental stages and tissue types. During germination, enzymes in peroxisomes catalyse β -oxidation and the glyoxylate cycle, thereby allowing lipids stored in seeds to be used as energy before photosynthetic machinery commences. As phototrophy is established several days after germination, peroxisomes are remodelled to carry out the glycolate pathway required for photorespiration. This remodelling most likely involves the simultaneous action of several processes, such as LON2-mediated degradation of the glyoxylate cycle enzymes and a specialized autophagy type called “pexophagy”. LON2 dysfunction results in glyoxylate cycle enzymes stabilization (Farmer et al. 2013; Goto-Yamada et al. 2014). Besides its protease activity, LON2 also has chaperone activity that suppresses pexophagy and peroxisome remodelling (Goto et al. 2014). Interestingly, the protease activity of LON2 seems to interfere with its chaperone-dependent inhibition of pexophagy and thus helps to control the protection of peroxisomes from autophagy. Remodelling of the peroxisomal proteome is also attributed to light- and sugar-dependent transcriptional changes, and possibly other proteases, the UPS and proteins involved in peroxisome biogenesis (Goto et al. 2015).

All examined *Arabidopsis lon2* mutant alleles are resistant to IBA-induced lateral root formation but respond normally to auxins not requiring β -oxidation (Lingard and Bartel 2009; Burkhart et al. 2012), suggesting reduced IBA-to-IAA conversion. Additionally, *lon2* mutants display age-dependent defects in PTS2 processing that are accompanied by defects in peroxisomal matrix protein import, indicating that LON2 is necessary for the sustained import of matrix proteins. Despite these defects in peroxisome physiology, disrupting *Arabidopsis* LON2 does not appear to result in matrix protein stabilization.

A second peroxisomal protease with a role in *Arabidopsis* matrix protein degradation is DEG15 (Schuhmann et al. 2012). The family of Deg proteases (for degradation of periplasmic proteins), also known as HtrA proteases (for high-temperature requirement A), are one important group of these proteolytic enzymes (Schuhmann et al. 2012). Degs are ATP-independent serine endopeptidases found in all domains of life, including Bacteria, Archaea and Eukarya. DEG15 processes PTS2 proteins into their mature forms by removing the Nt PTS2-containing (Helm et al. 2007; Schuhmann et al. 2008), but DEG15 is not required for isocitrate lyase or malate synthase degradation (Lingard and Bartel 2009). Similarly, the peroxisomal M16 metalloprotease PXM16 is not required for

degradation of glyoxylate cycle enzymes; *pxm16* and *lon2 pxm16* mutants efficiently degrade isocitrate lyase (Lingard and Bartel 2009).

Pexophagy is involved in peroxisomal quality control and removes damaged catalase aggregates and clustered peroxisomes (Kim et al. 2013; Shibata et al. 2013; Yoshimoto et al. 2014; Hackenberg et al. 2013). The plant pexophagy receptor for ATG8 remains elusive. A bioinformatics approach named hfAIM (high fidelity ATG8 interacting motif) identified 9 peroxisomal PEX proteins in *Arabidopsis* that contain putative hfAIM, among which PEX6 and PEX10 were further verified by bimolecular fluorescence complementation (Xie et al. 2016). Yeast two-hybrid screen identified PEX10 as an ATG8-interacting protein, suggesting that PEX10 is a promising candidate for a receptor in pexophagy (Marshall et al. 2019). A recent study showed that autophagy mediates glucose-promoted peroxisomal degradation in roots and that ATG8 physically interacts with a peptide of PXA1 (Peroxisomal ABC transporter 1)/CTS (Comatose)/PED3 (Peroxisome defective 3) (Huang et al. 2019), making PXA1 another possible receptor for pexophagy. Whether full-length PXA1 interacts with ATG8 in planta has not been shown. More rigorous studies will determine whether any of these ATG8-interacting proteins function as true pexophagy receptors.

6.4 Endoplasmic Reticulum

The ER environment is very sensitive and can result in cytotoxic conditions known as ER stress, which can compromise the balance between protein folding and synthesis. ER stress is under continuous surveillance and proteolytic pathways execute the removal of misfolded proteins. Processes including the unfolded protein response (UPR), ER-associated degradation (ERAD) and autophagy play important roles in restoring ER homeostasis. For excellent reviews on ERAD and UPR, we refer readers to Chen et al. (2020); Bao and Howell (2017).

In plants, we know two ER stress sensor branches: the inositol requiring enzyme 1 (IRE1) and the activating TF 6 (ATF6). IRE1 pathway orchestrates UPR and is the most evolutionary conserved branch of the UPR. The ATF6 (basic leucine zipper protein (bZIP)) are ER transmembrane proteins with a cytosolic Nt containing bZIP transcription factor domain and an ER C-terminal domain containing ER-retention signals (Vitale et al. 2019; Iwata et al. 2008). When cells encounter ER stress, the ATF6 complex translocates to the Golgi where it is activated via regulated intramembrane proteolysis (RIP) (Brown et al. 2000). RIP releases ATF6. This biological process is mediated via proteases and results in the release of the now active bZIPTF domain of the ATF6 complex to the cytosol which is then translocated to the nucleus, regulating ER stress responses (Pastor-Cantizano et al. 2020).

IRE1 modulates UPR in plants and is a single-pass transmembrane protein that has a luminal domain and cytoplasmic domain, conferring different functions at both ER membrane sides. IRE1 contains both RNase and protein kinase domains,

and the RNase activity, but not the protein kinase activity, of IRE1 is critical for ER stress-induced autophagy. In addition to clearing unfolded proteins under stress, ERAD also functions in the turnover of normal proteins to regulate cell development, such as HMG COENZYME A REDUCTASE (HMGCR) in mammals (Menzies et al. 2018). In development, ERAD functions seem conserved from mammals to plants. The mouse homolog of Ubc6p, UBE2J1, is required for spermiogenesis, evidenced by UBE2J1^{-/-} mice sterility (Koenig et al. 2014). Interestingly, in tomato (*Solanum lycopersicum*) SIUBC32 loss of function homolog of UBE2J1 results in the late-ripening of tomato fruit (Wang et al. 2014). Besides, in rice OsDER1 endosperm-specific knockdown induced UPR and resulted in abnormal phenotypes of seed size and starch granules. OsDER1 associates with OsHRD1, OsHRD3 and OsCDC48, suggesting that the HRD1-mediated ERAD pathway controls rice seed development (Qian et al. 2018).

ERAD may regulate auxin signalling. ER stress triggers the accumulation of AUX/IAA transcriptional repressors by downregulating auxin receptors and transporters in *Arabidopsis* (Chen et al. 2020). More research will determine ERAD involvement in other phytohormone-dependent growth regulation (Zhao et al. 2014). The same ERAD component, CER9/DOA10A/SUD1 (ECERIFERUM 9/DOA10A/SUPPRESSOR OF DRY2 DEFECTS1) that functions in both drought response and seedling development mediated by ABA signalling suggests that ERAD is essential in mediating the balance between plant growth and stress adaptation.

Furthermore, although selective degradation of ER by autophagy termed “ER-phagy” may function in plants, it is yet unclear whether it has direct roles in development. For more information on this topic, we refer readers to Bao and Bassham (2020). C53 protein is an ER-phagy receptor shared by plants and mammals. C53 harbours a unique shuffled ATG8 interacting motif (AIM) with ATG8 and is recruited to autophagosomes upon ER stress. C53 senses ER-associated stalled ribosomes and forms a tripartite receptor complex with the UFMylation ligase components UFL and DDRGK1, resulting in the degradation of nascent ER proteins. The corresponding *c53* mutants are therefore more ER stress-sensitive (Stephani et al. 2020). C53 thus provides an alternative quality control pathway for the maintenance of ER homeostasis.

7 Closing Remarks

The previous proteolytic pathways highlight proteases’ importance in the regulation of aspects of plant development (Fig. 2). The scarcity of information on relevant protease substrates is the major bottleneck in fully elucidating proteolytic pathways affecting development. Although proteolytic pathways have been traditionally linked to intrinsically destructive processes, we believe that this might be an oversimplification of a more profound and general role of such pathways throughout the life span of a cell. The endeavour of “de-orphanizing” proteases, finding their substrates may include high-resolution expression analyses and proteomics (e.g. as was done for the stomatal regulator SBT5.2 (Engineer et al. 2014)). Many proteases,

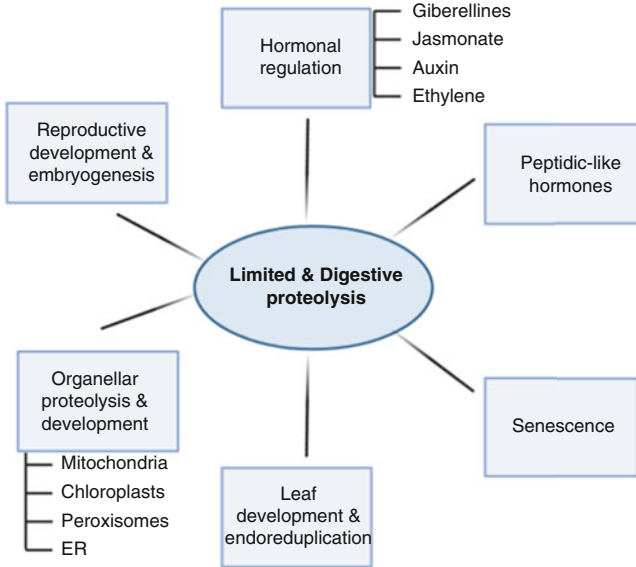


Fig. 2 Schematic representation of the most important developmental procedures, in which limited and digestive plant proteolysis are involved and described in brief in this chapter. Figure was created using BioRender (<https://biorender.com>)

however, are posttranslationally regulated, and therefore quantitative proteomics may not reveal their activity maxima. This problem can be solved by activity profiling methods with specific probes that can reveal the spatiotemporal protease activity (Pružinská et al. 2017). Moreover, expressing heterologous protease inhibitors from pathogens through careful selection of promoters may prove an elegant strategy to overcome genetic redundancies (e.g. SBTs in floral organ abscission; (Schardon et al. 2016)). Methods, such as positional proteomics, can be used to discover *in vivo* protease substrates and associated products of limited or digestive proteolysis. For example, the high-throughput proteomic approach dubbed COMBINED FRACTIONAL DIAGONAL CHROMATOGRAPHY (COFRADIC) revealed *in vivo* substrates of METACASPASE 9 (Wrzaczek et al. 2015; Tsiatsiani et al. 2011, 2012, 2013; Tsiatsiani and Heck 2015). Furthermore, information on protease localization is of paramount importance in inferring possible functions of proteases and relevance of the identified interactome, allowing researchers also to focus positional proteomics in a subset of the proteome, increasing the discovery rate of functionally relevant substrates. Such frameworks will streamline future efforts to decipher proteolytic pathways in an unthinkable depth, creating opportunities for practical applications.

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