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Review

Tansley insight

Life and death of proteins after protease cleavage: protein degradation by the N-end rule pathway

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Summary

The N-end rule relates the stability of a protein to the identity of its N-terminal residue and some of its modifications. Since its discovery in the 1980s, the repertoire of N-terminal degradation signals has expanded, leading to a diversity of N-end rule pathways. Although some of these newly discovered N-end rule pathways remain largely unexplored in plants, recent discoveries have highlighted roles of N-end rule-mediated protein degradation in plant defense against pathogens and in cell proliferation during organ growth. Despite this progress, a bottleneck remains the proteome-wide identification of N-end rule substrates due to the prerequisite for endoproteolytic cleavage and technical limitations. Here, we discuss the recent diversification of N-end rule pathways and their newly discovered functions in plant defenses, stressing the role of proteases. We expect that novel proteomics techniques (N-terminomics) will be essential for substrate identification. We review these methods, their limitations and future developments.

I. Introduction: conservation and diversity of N-end rule pathways

The control of protein stability plays a key role in the regulation of all cellular processes and, in eukaryotes, is largely controlled by the ubiquitin–proteasome system (UPS). The N-end rule pathway, a subset of the UPS, relates the *in vivo* half-life of a protein to the nature of its N-terminal amino acid residue and some of its post-translational modifications (PTMs). Removal of the initiator Met residue by methionine aminopeptidases (MetAPs) or cleavage of pre-proproteins (i.e. proteins that bear signal peptides and/or that require cleavage for their activation or degradation) by endoproteases exposes new N-terminal residues, potentially directing the resulting protein fragments for degradation by the N-end rule pathway (Fig. 1). In eukaryotes, the N-end rule pathway comprises different branches that mediate the degradation

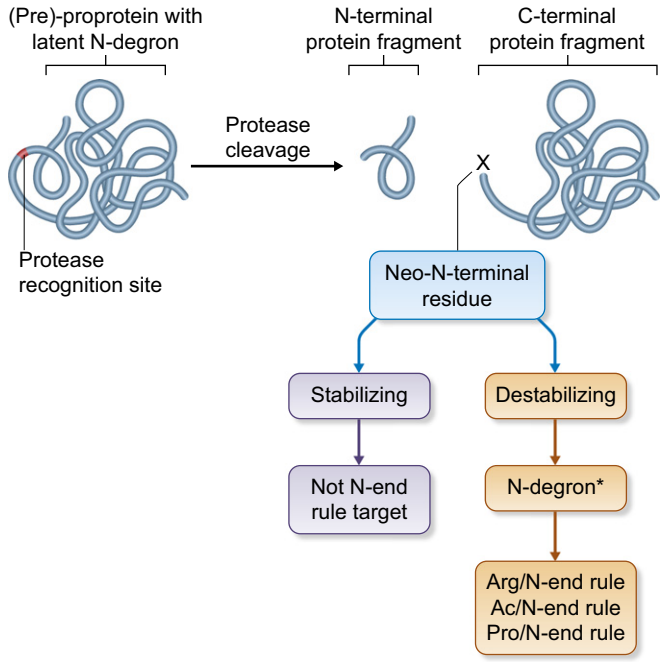

Fig. 1 Role of proteases in the generation of N-end rule substrates. Endoproteolytic cleavage of a pre-proprotein results in the exposure of a new N-terminal or 'neo-N-terminal' residue. This endoproteolytic event can expose to the solvent a 'latent' or 'dormant' N-terminal degradation signal, or N-degron, that was previously buried in the internal sequence of the protease recognition site. Neo-N-terminal residues may be either 'stabilizing' or 'destabilizing' based on the Arg/ or Ac/ or Pro/N-end rule and may hence serve to target the protein fragment for degradation by the N-end rule pathway. However, not all N-terminal destabilizing residues lead to a functional N-degron (denoted by an asterisk) and degradation of the target protein. Additional structural and sequence features, such as flexibility of the N-terminal region, presence of Lys side chains as ubiquitin acceptor sites and charge or hydrophobicity of residues close to the neo-N-terminal, are also critical for an N-end rule target. Furthermore, the N-terminal methionine residue of a protein may serve directly as a destabilizing residue, so that endoproteolytic cleavage is not always a prerequisite.

of proteins whose N-terminal residues are acetylated (Ac/N-end rule) and non-acetylated, respectively (reviewed by Varshavsky, 2011; Gibbs *et al.*, 2016). Together, these two branches of the N-end rule pathway can recognize all existing N-terminal amino acid residues and some of their modifications. This includes the initiator Met residue that is typically found at the N-terminus of proteins (Hwang *et al.*, 2010; Kim *et al.*, 2014).

Recent findings have led to an expansion of the acetylation-independent branch, which now comprises the 'classic' Arg/N-end rule (described in more detail below) and the newly found Pro/N-end rule pathway (Chen *et al.*, 2017). The latter was discovered in yeast and targets for degradation proteins with Pro at first or second position through the activity of Gid4, a subunit of the GID ubiquitin ligase (Santt *et al.*, 2008) that targets gluconeogenic enzymes in yeast (Hammerle *et al.*, 1998; Chen *et al.*, 2017). While it is not yet known whether the Pro/N-end rule is present in multicellular eukaryotes, components of the Arg/ and Ac/N-end rule pathways appear to be mostly conserved (reviewed by Varshavsky, 2011; Tasaki *et al.*, 2012; Lee *et al.*, 2016). For example, the hierarchical organization of the Arg/N-end rule pathway is overall the same in eukaryotes (Fig. 2): N-terminal primary destabilizing residues can be directly bound by E3 ligases called N-recognins; secondary destabilizing residues require conjugation of Arg (a primary destabilizing residue) by the conserved Arg-transferases (ATEs); and tertiary destabilizing residues are first enzymatically or chemically transformed into secondary destabilizing residues before arginylation by ATEs.

The evolutionary conservation of different N-end rule pathways is further underlined by the recent suggestion that chloroplasts (Rowland *et al.*, 2015; Zhang *et al.*, 2015) and mitochondria (Vogtle *et al.*, 2009; Calvo *et al.*, 2017) might also have organelle-specific N-end rule pathways that resemble that of prokaryotes.

II. Defensive functions of the N-end rule pathway in plants

While the recent diversification of N-end rule pathways in eukaryotes has expanded the repertoire of destabilizing residues and modifications that serve as N-terminal degradation signals (or N-degrons), in plants, functions of the Ac/ and Arg/N-end rule pathways are just emerging. In particular, these pathways have been involved in plant responses to a variety of developmental and environmental signals (reviewed by Gibbs *et al.*, 2014, 2016; Lee *et al.*, 2016). The most recent discoveries include their role in the control of plant defense responses. First, the stability of the Nod-like immune receptors (NLRs) SUPPRESSOR OF NPR1, CONSTITUTIVE1 (SNC1) and RESISTANCE TO *Pseudomonas syringae* pv. *maculicola*1 (RPM1) is regulated through N-terminal acetylation (Xu *et al.*, 2015). It is tempting to speculate that the Ac/N-end rule pathway might participate in NLR homeostasis, as NatA-mediated acetylation of the first Met residue of SNC1 contributes to its degradation. Second, a new role for the Arg/N-end rule pathway was uncovered in activating the production of defense-related metabolites, such as glucosinolates and the phytohormone jasmonic acid (de Marchi *et al.*, 2016). It was additionally shown that the Arg/N-end rule pathway positively regulates defenses against a wide range of bacterial and fungal pathogens with different lifestyles and, more particularly, that the ATEs regulate the timing and amplitude of the defense program against avirulent bacteria (de Marchi *et al.*, 2016). That study also led to the discovery of the first physiological function of the PRT1 N-recognin, which appears to act as a positive regulator of plant defenses (de Marchi *et al.*, 2016). Third, a recent report highlighted a link between the known functions of the Arg/N-end rule pathway in the degradation of key transcriptional regulators of the hypoxia response (the ERFVII transcription factors; Gibbs *et al.*, 2016) and *Arabidopsis* infection by the protist *Plasmodiophora brassicae*, which triggers clubroot development (Gravot *et al.*, 2016). Their study suggests strongly that Arg/N-end rule-driven hypoxia responses may be a general feature of pathogen-induced gall development in plants.

III. Proteases and degradation by the N-end rule pathway

Despite recent progress in our understanding of the Ac/ and Arg/N-end rule pathways in plants, elucidation of the underlying

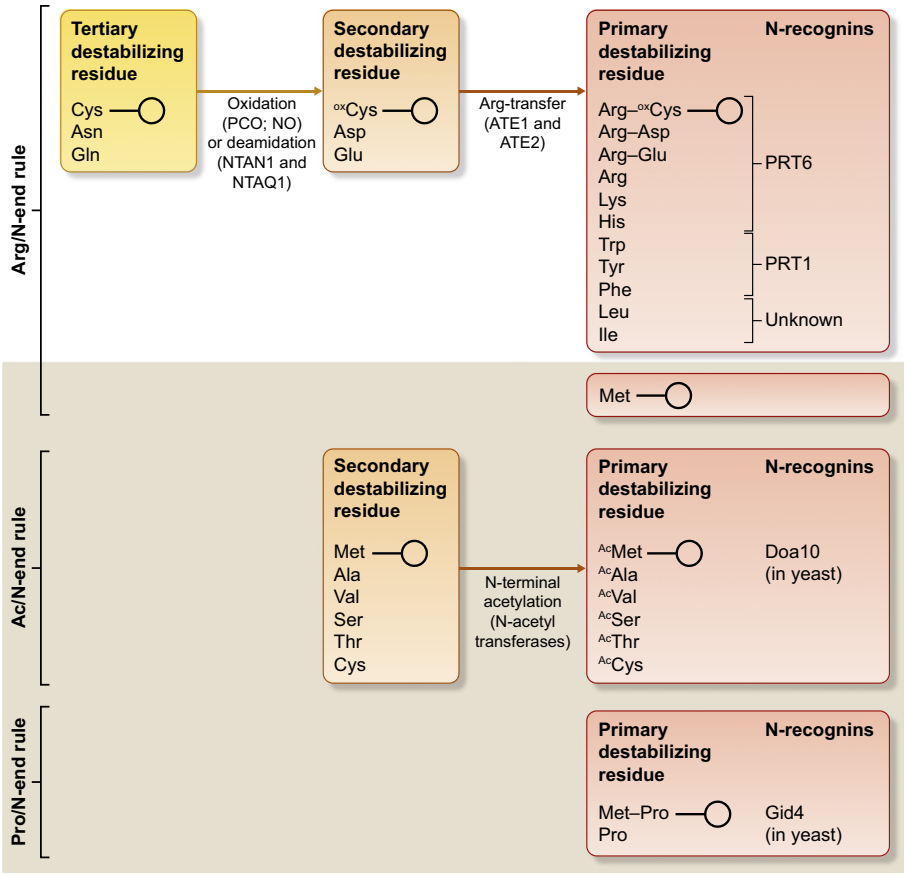

Fig. 2 Canonical N-end rule pathways. The neo-N-terminus of a protein may be a tertiary (yellow), secondary (orange) or primary (red) destabilizing residue. One potential outcome of the exposure of neo-N-termini is their modification involving deamidation or Cys oxidation, arginylation, acetylation and finally ubiquitylation, followed by degradation of the protein by the Arg/, Ac/ or Pro/N-end rule pathways. Protein fragments starting with primary destabilizing residues can be recognized and bound by so-called N-recognins, which belong to the class of E3 ubiquitin ligases. *In vivo* studies with artificial N-end rule reporter substrates in plants show that PRT6 in specific for positively charged or Type 1 residues (Garzon *et al.*, 2007) and that PRT1 recognizes aromatic hydrophobic or Type 2 residues (Potuschak *et al.*, 1998). Furthermore, *in vitro* ubiquitylation assays of fluorescently labeled artificial N-end rule substrates confirmed the specificity of PRT1 and its E3 ligase activity (Mot *et al.*, 2018). Note that the degradation of proteins with N-terminal Met, as well as through the Ac/ and Pro/N-end rule (all represented on a beige background) have not yet been demonstrated to exist in plants, but are found in yeast (Hammerle *et al.*, 1998; Chen *et al.*, 2017). In yeast, the Ac/N-end rule requires the activity of the E3 ligase Doa10. The Ac/N-end rule is also found in animals (Hwang *et al.*, 2010; Varshavsky, 2011). Circles denote the C-terminal protein fragments after endoproteolytic cleavage. NO, nitric oxide; PCO, PLANT CYSTEINE OXIDASE.

molecular mechanisms has remained largely elusive due to technical difficulties for the proteome-wide identification of N-end rule substrates and the complex mechanisms that lead to their formation. Indeed, most Arg/N-end rule substrates identified in yeast and animals are generated through protease cleavage (Figs 1, 2; Rao *et al.*, 2001; Ditzel *et al.*, 2003; Piatkov *et al.*, 2012a,b; Brower *et al.*, 2013; reviewed by Tasaki *et al.*, 2012), making it difficult to predict N-end rule substrates without detailed knowledge of protease cleavage sites and substrates. Moreover, an N-terminal destabilizing residue is not necessarily sufficient for the generation of an N-degron. Indeed, the accessibility of the N-terminal residue for N-recognin binding, the properties of the residues neighboring the N-terminus and the proximity of a Lys residue that may be ubiquitylated are also important (Tasaki *et al.*, 2012; Wadas *et al.*, 2016; Mot *et al.*, 2018). The complexity of an N-degron probably explains why many proteins with a presumed N-terminal destabilizing residue appear to be relatively abundant and stable in plant cells (Li *et al.*, 2017).

As highlighted by two recent proteomics studies, endoproteolytic events that can lead to N-end rule-mediated degradation of protein fragments are prevalent in plant cells. The first study (Zhang *et al.*, 2015) used quantitative proteomics to identify N-end rule substrates that were expected to accumulate in Arg/N-end rule mutants compared to the wild type. The second study (Venne *et al.*, 2015) aimed at advancing techniques to characterize and quantify neo-N-termini for dissecting proteolytic events. Despite using different methods, the majority of N-terminal fragments identified in these studies were the result of a protease cleavage or of initiator Met excision, suggesting that most intracellular proteins are endoproteolytically processed. However, most of the newly exposed N-terminal residues were not destabilizing based on the Arg/N-end rule, suggesting that: (1) many proteolytic fragments are not substrates of the Arg/N-end rule pathway; and (2) fragments starting with destabilizing residues may not be detected, possibly because of their rapid Arg/N-end rule-dependent degradation. Moreover, N-terminal

acetylation accounted for 55% of the N-terminal fragments identified, with most of these appearing to be acetylated co-translationally. These results hence also highlight the potential relevance of the Ac/N-end rule pathway in plants (Zhang *et al.*, 2015).

How can N-end rule substrates be identified given the prerequisite for protease cleavage? One possibility is to use knowledge of protease substrates and cleavage sites. The latest example of an N-end rule substrate identified using such information is that of the organ-size regulator BIG BROTHER, which is cleaved by the protease DA1 in Arabidopsis. The resulting C-terminal fragment then appears to be targeted for degradation by the N-recognin PRT1 (Dong *et al.*, 2017). It is also worth noting that the recently discovered defensive functions of the Ac/ and Arg/N-end rule pathways are coherent with a potential role of defense-related plant proteases (including MetAPs) in generating N-end rule substrates. One example highlighting the connection between proteases and the generation of N-end rule substrates during plant–pathogen interactions is the cleavage of the central *Arabidopsis* defense regulator RPM1-INTERACTING PROTEIN4 (RIN4) by the *P. syringae* protease effector AvrRpt2, which leads to RIN4 fragments with potential N-terminal destabilizing residues (Chisholm *et al.*, 2005; Eschen-Lippold *et al.*, 2016). Although no *in vivo* evidence has been provided to date, it has been suggested that AvrRpt2-derived RIN4 fragments could be degraded by the Arg/N-end rule pathway (Takemoto & Jones, 2005). Yet another example is the list of potential N-end rule substrates generated following cleavage by METACASPASE9 in *Arabidopsis* (Tsiatsiani *et al.*, 2013). However, similarly to RIN4, *in vivo* evidence that any of these fragments are degraded by the N-end rule pathways is still lacking.

Other putative N-end rule substrates may be predicted using the primary sequence of proteins starting with Met-Cys, as MetAPs may excise the initial Met residue, exposing the Cys at the N-terminus of the protein. The resulting N-terminal Cys residue may then be oxidized through either a chemical reaction or the activity of Cys oxidases (Fig. 2). The latter, termed PLANT CYSTEINE OXIDASEs (PCOs), have so far only been found in plants (Weits *et al.*, 2014) and generate N-terminal Cys sulfinic acid (White *et al.*, 2016), which can act as an N-degron.

Finally, novel developments that further highlight the role of proteases in the generation of N-end rule substrates in plants include the potential existence of a chloroplast-specific N-end rule (Nishimura & van Wijk, 2015). Indeed, recent studies show that following cleavage of the chloroplast transit peptide, destabilizing residues (of the prokaryotic N-end rule pathway) are under-represented in nuclear-encoded chloroplast proteins (Rowland *et al.*, 2015; Zhang *et al.*, 2015). Together with the recent discovery of a chloroplast ortholog of the bacterial ClpS N-recognin (Nishimura *et al.*, 2013), these results suggest that stromal-processing peptidases may play a role in the generation of chloroplast N-end rule substrates (Rowland *et al.*, 2015). Strikingly, a mitochondrion-specific N-end rule with similarities to the prokaryotic N-end rule could also exist (Vogtle *et al.*, 2009; Calvo *et al.*, 2017).

IV. New proteomics approaches for the identification of N-end rule substrates

Given the pre-eminent role of proteases in the generation of N-end rule substrates, recent attempts at discovering new N-end rule substrates in plants have relied on novel proteomics techniques, termed N-terminomics, that were initially developed to characterize proteolytic events and identify newly exposed neo-N-terminal residues and their PTMs (e.g. acetylation, oxidation, deamidation, arginylation) (Fig. 3). These novel N-terminomics techniques address some of the limitations of shotgun proteomics approaches, which had been developed to compare global protein abundance (e.g. in a wild-type vs a mutant plant), but could not provide information on the identity of N-terminal residues and potential N-end rule substrates (Majovsky *et al.*, 2014).

N-terminomics approaches are based on targeted enrichment of N-terminal peptides through chemical labeling of α-amine groups of N-terminal residues, which makes them distinguishable from internal amines derived from sample treatment by proteases (Huesgen & Overall, 2012). This specific N-terminal labeling reduces the complexity of the peptide mixtures and allows the identification of the true N-termini of mature proteins. N-terminomics approaches use various strategies to separate N-terminal peptides from internal ones. COmbined FRActional DIagonal Chromatography (COFRADIC; Gevaert *et al.*, 2003) and Charge-based FRActional DIagonal Chromatography (ChaFRADIC; Venne *et al.*, 2013) rely on different chromatographic techniques to enrich for N-terminal peptides. Terminal Amine Isotopic Labeling of Substrates (TAILS; Kleifeld *et al.*, 2010; Rowland *et al.*, 2015; Zhang *et al.*, 2015) allows the capture of N-terminal peptides via chemical modification. Other techniques include Stable-Isotope Protein N-terminal Acetylation Quantification (SILProNAQ; Bienvenut *et al.*, 2015) and Proteomic Identification of protease Cleavage Sites (PICS; Schilling *et al.*, 2011). Importantly, these techniques can be coupled with immunoprecipitation approaches, for example using antibodies raised against artificial peptides harboring specific destabilizing N-terminal residues, to further enrich samples for N-end rule substrates (Hoernstein *et al.*, 2016). As these techniques develop further, we expect that they will greatly contribute to our understanding of the molecular mechanisms underlying the functions of the N-end rule pathway in plants.

V. Concluding remarks

How the regulation of protein stability contributes to developmental processes and to plant responses to environmental cues remains a key question. In recent years, the N-end rule pathway has emerged as an important regulator of these processes. Despite this progress, the number of known N-end rule substrates remains small, largely due to the complex proteolytic mechanisms that lead to their formation. Indeed, specific endogenous or exogenous triggers, such as a stress or developmental cues, that lead to endoproteolytic cleavage and exposure of N-terminal destabilizing residues are often required to generate N-end rule substrates. Hence, these substrates might only be generated in specific

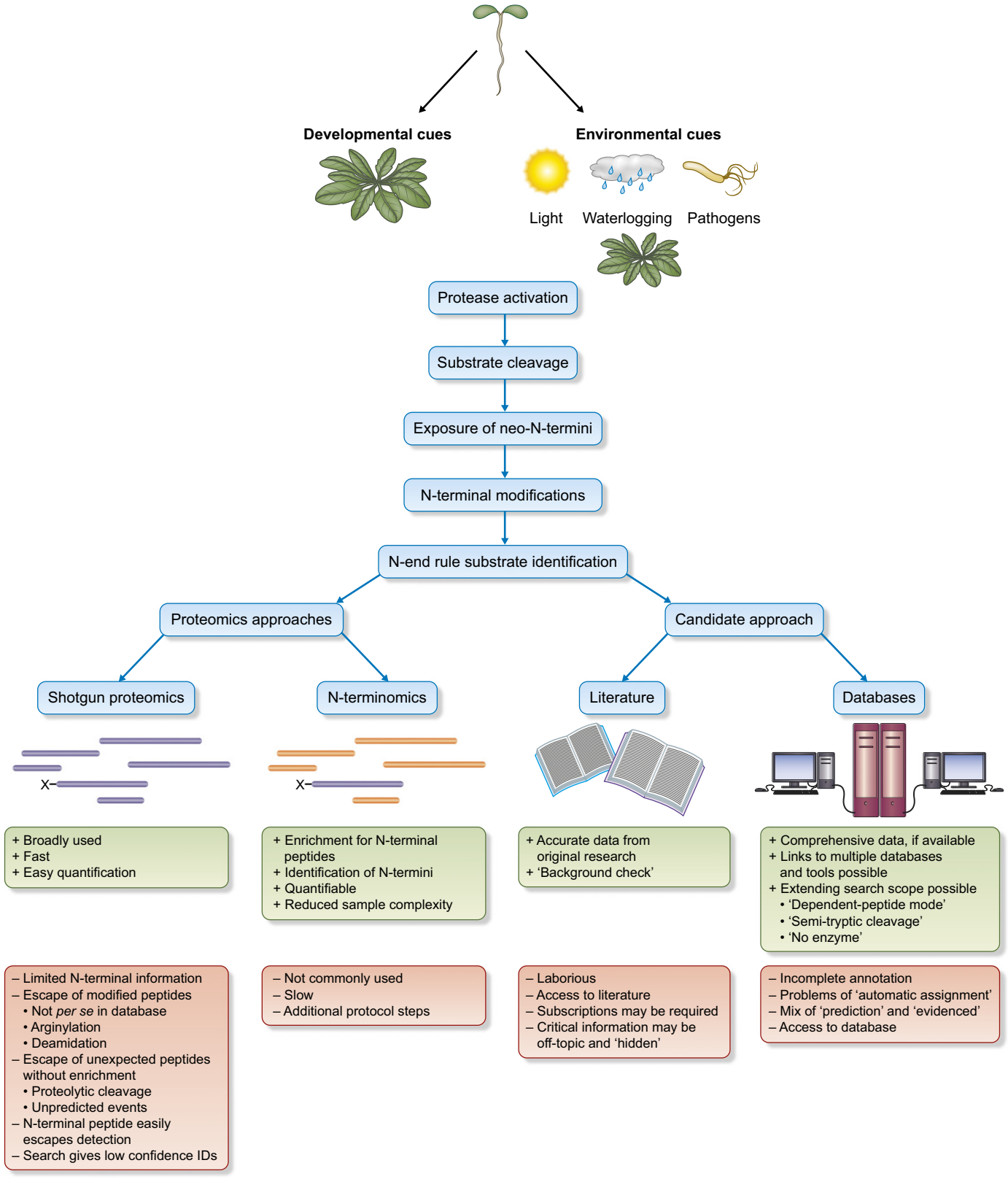

Fig. 3 Approaches used to identify N-end rule substrate candidates. Endoproteolytic cleavage of (pre-)proproteins plays an important role in the generation of N-end rule substrates. The activity of proteases is tightly regulated and may depend on both developmental and environmental cues. Hence, specific N-end rule substrates may exist only in specific conditions or at specific developmental stages. As primary protein sequence information alone is mostly insufficient to predict N-end rule substrates, more complex proteomics methods have been recently applied. These include shotgun proteomics and more specific N-terminomics approaches. In addition, scanning through the literature or searching through databases can lead to the identification of putative N-end rule substrates. Pros and cons of the different methods are highlighted in green and red, respectively.

conditions, and probably accumulate in a transient and/or in a cell type-specific manner. Other key limitations include the lack of knowledge of protease cleavage sites and resulting neo-N-termini in plants, as well as technical limitations of proteomics approaches. For example, insufficient sensitivity of current proteomics methods and protocols, together with the nature and low abundance of the PTMs to be detected (i.e. acetylation, oxidation, deamidation, arginylation) have hampered N-end rule substrate discovery. Method optimization for the identification of N-end rule substrates includes the use of various proteases as alternatives to trypsin, which cleaves after Arg. Improving search algorithms that allow us to identify all possible peptides, including unusual ones with mass increments corresponding to specific PTMs, is also essential. Potential loss of information can also be counteracted by adapting search modes to identify more accurately protein fragments that may result from unpredicted cleavage by unknown proteases present in the proteome. We expect that improved proteomics methods will allow the direct identification of proteins with N-terminal destabilizing residues, while also increasing the completeness and accuracy of databases for protease cleavage sites, further facilitating the identification of N-end rule substrates.

In summary, the N-end rule pathway represents a central and emerging field of investigation to understand the role of protein degradation in plants. Importantly, it also has a potential for applications in agronomy. For example, it has been shown that mutants of ATEs or PRT6 accumulated ERFVII transcription factors that act as master regulators of the hypoxia response. This accumulation correlated with increased tolerance to waterlogging (Gibbs *et al.*, 2011, 2016; Riber *et al.*, 2015; Mendiondo *et al.*, 2016). Furthermore, easy manipulation of turnover rates of recombinant target proteins by using temperature-inducible N-degrons (Faden *et al.*, 2016) indicates that the N-end rule pathway may also be a valuable tool for biotechnological applications in the future.

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See also the Commentary on this article by van der Hoorn & Rivas, **218**: 879–881.