The plant N-end rule pathway: structure and functions

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The N-end rule pathway is a protein degradation pathway that relates the stability of a protein to the nature of its N-terminal amino acid residue. This pathway is part of the ubiquitin–proteasome system in eukaryotes and has been shown to be involved in a multitude of cellular and developmental processes in animals and fungi. However, in plants, its structure and functions have long been enigmatic. In this review, we discuss recent advances in the identification of the enzymatic components that mediate protein degradation through the N-end rule pathway in plants. We further describe the known functions of this pathway in the control of plant growth and development and outline open questions that will likely be the focus of future research.

Discovery and structure of the N-end rule pathway

The half-life of cellular proteins varies greatly, ranging from a few seconds to several days [1,2]. In eukaryotes, protein stability is largely controlled by the ubiquitin–proteasome system (UPS), which catalyzes the conjugation of the 8-kDa protein ubiquitin (Ub) to Lys residues of substrate proteins, marking them for proteolysis by the 26S proteasome [3]. Regulated proteolysis by the UPS underlies almost every important cellular and organismal function in eukaryotes. In plants, Ub-dependent processes play major and diverse roles, for example, in regulating the signaling by phytohormones such as auxin, gibberellins and jasmonic acid (reviewed in [4]).

The conjugation of Ub to substrate proteins requires the activity of three types of enzymes: E1 Ub-activating enzymes, E2 Ub-conjugating enzymes, and E3 Ub ligases [5,6]. E3 Ub ligases recognize and bind specific degradation signals in substrate proteins and thus confer specificity to the UPS. The first degradation signal, or degron, was discovered in the 1980s in the laboratory of Alexander Varshavsky [7–9]. Andreas Bachmair *et al.* [7,8] expressed fusion proteins in the yeast *Saccharomyces cerevisiae* that comprised an N-terminal Ub followed by a junctional amino acid residue ('X'), which varied in the different fusion proteins, and the reporter protein β-galactosidase (β-Gal) at the C-terminus (Figure 1). They observed that the N-terminal Ub moiety was cleaved off the fusion protein after the last residue of Ub (a process later shown to be catalyzed by deubiquitylating enzymes [10]). This deubiquitylation yielded a β-Gal reporter protein with the junctional residue X at its N-terminus. Bachmair *et al.* further noted that, whereas some N-terminal residues (termed stabilizing

residues; see Glossary) caused accumulation of β-Gal, other so-called destabilizing residues resulted in low reporter levels. The differences in β-Gal accumulation were found to correlate with different half-lives of the reporter proteins [7]. These findings implied the existence of a protein degradation pathway that relates the *in vivo* stability of a protein to the nature of its N-terminal residue. This pathway was aptly named 'N-end rule pathway'.

A detailed characterization of the N-end rule pathway was initially carried out in fungi and animals [7,8,11,12], which revealed that it is part of the UPS and that it has a hierarchical organization (Figure 2; detailed below). Furthermore, it was demonstrated that the degron responsible for targeting a protein for degradation by the N-end rule pathway (termed N-degron) not only comprises a destabilizing N-terminal residue, but also requires a Lys residue for Ub conjugation in spatial proximity to the N-terminus, as well as a flexible region in the vicinity of these two determinants [8,13,14].

In contrast to fungi and animals, the characterization of the N-end rule pathway in plants has only recently come of

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Glossary

Destabilizing residue: an N-terminal residue that triggers degradation of a protein by the N-end rule pathway, but only when additional determinants are also present (see definition of N-degron).

N-degron: motif found in N-end rule substrates that is recognized by N-recognins. In eukaryotes, an N-degron is composed of an N-terminal destabilizing residue, as well as a neighboring Lys residue that serves as an attachment site for multi-Ub chains. The exposure of the N-degron also depends on the presence of a flexible region in the vicinity of the determinants.

N-end rule pathway: a protein degradation pathway that relates the *in vivo* stability of a protein to the nature of its N-terminal residue.

N-recognin: E3 Ub ligase of the N-end rule pathway that recognizes N-degrons and targets substrate proteins for degradation.

Primary destabilizing residue: an N-terminal residue that can be directly recognized by an N-recognin. Primary destabilizing residues fall into two categories: basic (type 1) and bulky hydrophobic (or type 2).

Secondary destabilizing residue: proteins with such N-terminal residues are first modified by aminoacyl-transferases, which conjugate a primary destabilizing residue to the N-terminus of the substrate protein. Alternatively, in yeast, a secondary destabilizing residue can be acetylated, which results in its recognition by the Doa10 Ub ligase.

Stabilizing residue: an N-terminal residue that prevents degradation of the protein by the N-end rule pathway. However, a protein starting with a stabilizing residue might still have a short half-life, if other degrons are present.

Tertiary destabilizing residue: N-terminal residues have to be first enzymatically or chemically modified before the substrate proteins can be arginylated and then recognized by N-recognins.

Ub fusion technique: a technique that allows the expression of proteins starting with a specific N-terminal residue ('X'). To this end, a fusion of an N-terminal Ub molecule, followed by a varying residue X and a reporter protein (e.g. Ub–X–βGal) is expressed in the cell. Deubiquitylating enzymes remove the N-terminal Ub moiety co-translationally, resulting in a reporter protein with a specific N-terminal residue.

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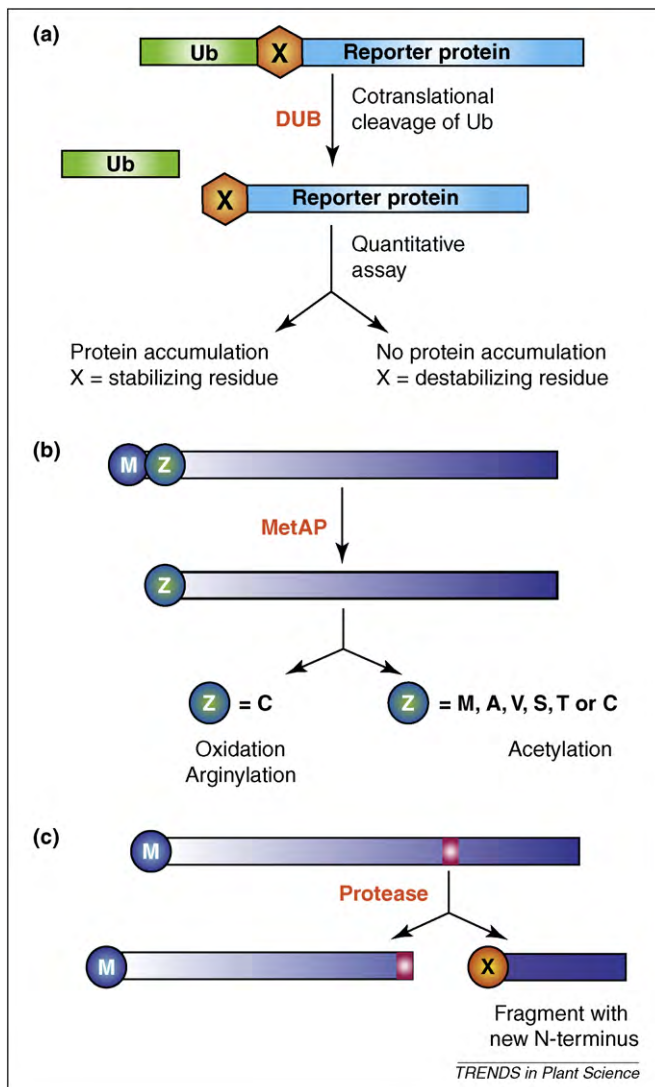
Figure 1. The Ub fusion technique and N-end rule substrates. N-terminal residues are indicated by single-letter abbreviations. **(a)** Artificial N-end rule substrates are generated through the Ub fusion technique. Expression of a Ub-X-reporter fusion results in its co-translational cleavage by deubiquitylating enzymes (DUBs) and the release of a X-reporter protein bearing a specific residue X at its N-terminus. At steady-state levels, quantitative measurements of the amounts of the reporter protein are representative of reporter protein stability [8,83]. **(b)** N-end rule substrates formed after MetAP cleavage. Depending on the nature of the second residue Z, the protein can be targeted for degradation by the N-end rule pathway through two different mechanisms: either oxidation and arginylation, or acetylation. **(c)** Generation of N-end rule substrates by proteolytic cleavage. Specific protein signals can be recognized and cleaved by a protease. This results in the release of a fragment with a new N-terminal residue. In cases where such cleavage exposes an N-degron, this fragment is targeted for degradation by the N-end rule pathway.

age. In this review, we first summarize the current knowledge about the structure of the plant N-end rule pathway and its enzymatic components. We then discuss the known functions of this pathway in the control of plant development and outline key questions that are currently unanswered and that will likely be the focus of research in coming years.

The identification of the complete sets of stabilizing and destabilizing residues in plants [15–19], as well as of some of its enzymatic components [17,19–21], showed that the organization of the plant N-end rule pathway is largely identical to that of animals. In plants, as well as in most

organisms examined, different types of destabilizing N-terminal residues have been identified (Figure 2) [12,19]. If made N-terminal, primary destabilizing residues are recognized and bound by E3 Ub ligases of the N-end rule pathway [12,22–24], termed N-recognins. Primary destabilizing residues can be further subdivided into two groups: type 1 or basic residues (Arg, Lys and His), and type 2 or bulky hydrophobic residues (Phe, Trp, Tyr, Leu and Ile).

In contrast to N-end rule substrates with primary destabilizing residues, proteins with secondary destabilizing residues have to be first enzymatically altered before they are targeted for degradation (Figure 2). Substrates bearing N-terminal Asp, Glu or oxidized Cys are modified by an arginyl-tRNA-protein transferase (R-transferase), which conjugates Arg to their N-terminus. Because Arg is a primary destabilizing residue, this modification results in the recognition of the proteins by an N-recognin [11,25–27].

Finally, proteins starting with tertiary destabilizing residues are modified before they are arginylated by R-transferases and recognized by N-recognins (Figure 2). N-terminal Asn is deamidated into the secondary destabilizing residue Asp by an enzyme termed N-terminal^{Asn} amidase (noted Nt^N-amidase) [28], whereas Gln is deamidated into Glu by the Gln-specific Nt^Q-amidase [19,29]. In animals and probably also in plants, N-terminal Cys requires oxidation through a chemical reaction involving nitric oxide and oxygen before it can be modified by R-transferases [19,30,31].

An alternative route for the generation of N-end rule substrates, which might also exist in animals and plants, has recently been uncovered in yeast. It was found that proteins starting with Met, Ala, Val, Ser, Thr and Cys can be acetylated *in vivo*, which leads to their recognition by the Ub ligase Doa10 and degradation by the 26S proteasome [32]. This work has provided exciting new insights into the generation of substrates of the N-end rule pathway, almost 25 years after its initial discovery.

Evolutionary conservation of the N-end rule pathway

The N-end rule pathway is present in prokaryotes [33–35] and eukaryotes. Despite the absence of a UPS in bacteria, the N-end rule pathways of prokaryotes and eukaryotes show striking similarities. For example, prokaryotic and eukaryotic N-recognins share substrate recognition domains, suggesting a common evolutionary origin [36,37]. Similarities among the N-end rule pathways of prokaryotes and eukaryotes also extend to aminoacyl-transferases responsible for the modification of the secondary destabilizing residues Asp and Glu (Figure 2) [38].

Among eukaryotes, the hierarchical organization of the N-end rule pathway is largely conserved and differences are mainly found at the level of its enzymatic components. For example, contrary to yeast, whose genome encodes a single bifunctional Nt-amidase that deamidates both N-terminal Asn and Gln [39], the genomes of plants and animals [28,29] code for two distinct Nt-amidases specific for either N-terminal Asn or Gln [19] (see above). Notably, the plant and animal Nt^N- and Nt^Q-amidases are not related in sequence to each other or to the yeast Nt^{N,Q}-amidase [28,29].
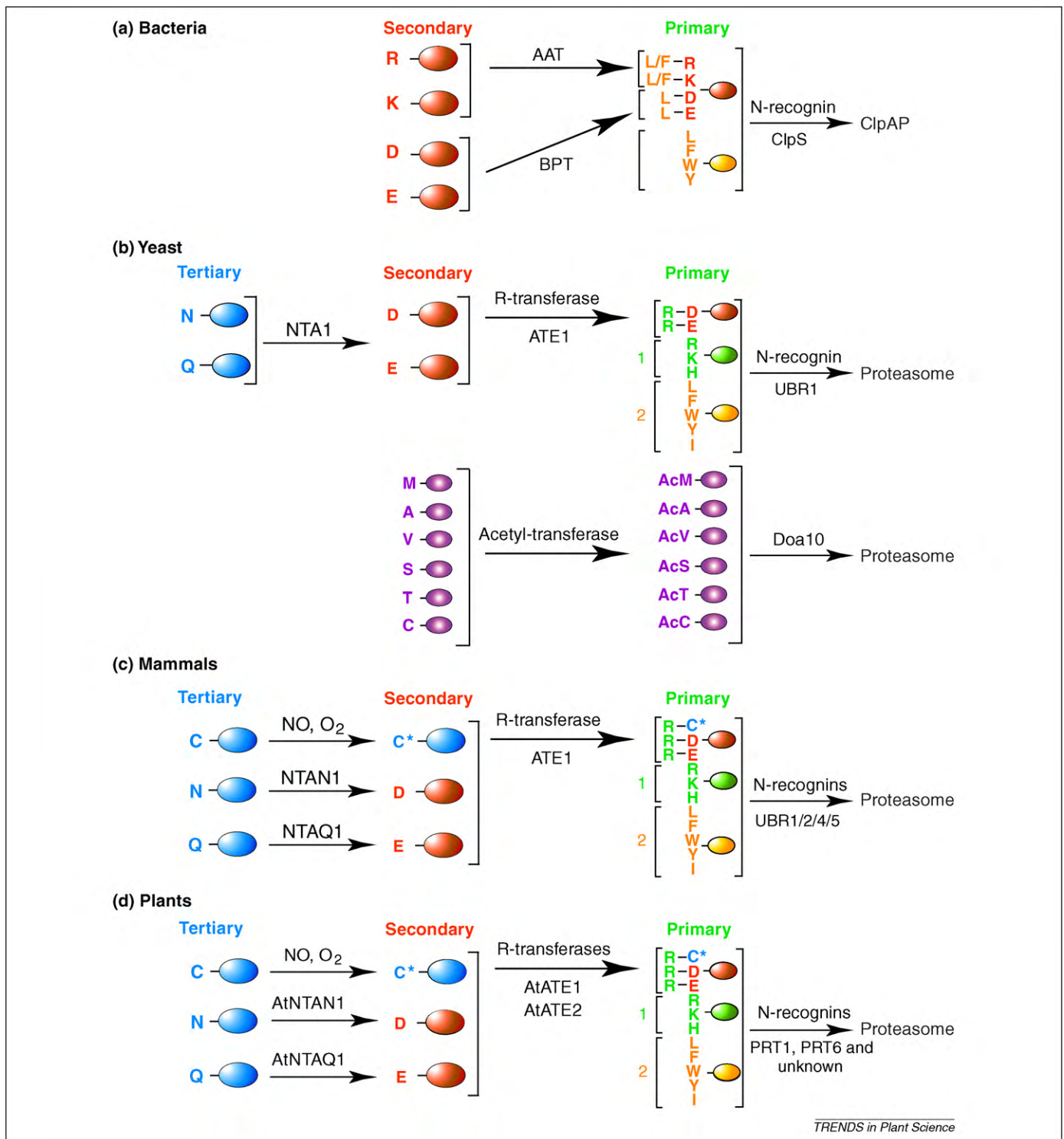
Figure 2. The N-end rule pathway in bacteria, fungi, mammals and plants. Ovals represent a protein substrate. N-terminal residues are indicated by single-letter abbreviations. C* denotes oxidized N-terminal Cys. **(a)** In bacteria, proteins with N-terminal primary destabilizing residues are bound by the N-recognin ClpS, which results in their degradation by the ClpAP protease [34]. Proteins starting with secondary destabilizing residues can be modified by two different aminoacyl-tRNA-protein transferases termed AAT and BPT [38]. **(b)** In the yeast *S. cerevisiae*, tertiary destabilizing residues are deamidated by a single Nt^{N,Q}-amidase (NTA1), whereas the R-transferase ATE1 recognizes proteins with the secondary destabilizing residues Asp and Glu and conjugates Arg to their N-termini. The yeast genome encodes a single N-recognin, UBR1, which binds to type 1 (basic) and type 2 (bulky hydrophobic) residues. Certain N-terminal acetylated residues also act as a degradation signal after recognition of the substrate protein by the Doa10 Ub ligase [32]. **(c)** The N-end rule pathway in mammals is similar to that of yeast, but does exhibit some differences. For example, two distinct Nt-amidases (NTAN1 and NTAQ1) are involved in the deamidation of N-terminal Asn and Gln, respectively [29,62]. In mammals, Cys is an additional tertiary destabilizing residue that requires oxidation through a chemical reaction involving nitric oxide (NO) and oxygen (O_2), prior to its recognition by the R-transferase ATE1 [30,31]. Contrary to yeast, the genomes of mammals encode several N-recognins (UBR1, UBR2, UBR4 and UBR5) [24]. **(d)** The hierarchical organization of the N-end rule pathway in plants is similar to that found in mammals [19]. However, only two N-recognins, PRT1 and PRT6 [17,41], have been identified to date.

Additional differences between the N-end rule pathways of fungi, animals and plants are found at the level of the N-recognins. Whereas the yeast *S. cerevisiae* has a single N-recognin (UBR1) [22], at least four different N-recognins (UBR1, UBR2, UBR4 and UBR5) are encoded by mammalian genomes [24]. All yeast and mammalian N-recognins identified so far contain a UBR domain, a zinc-finger-like motif of ~70 residues [24] that is sufficient for the binding of basic destabilizing residues [24,36,40]. Like animals, the genomes of plants encode several N-recognins, two of which have been identified in the model plant *Arabidopsis thaliana* and named PROTEOLYSIS1 (PRT1) and PROTEOLYSIS6 (PRT6) [17,18,20,41]. Notably, one of these N-recognins, PRT1, has no sequence similarity to mammalian or fungal N-recognins and does not contain a UBR domain [19,20] even though it recognizes similar sets of destabilizing residues. The uniqueness of PRT1 suggests that some N-recognins might have evolved after the plant and animal lineages diverged.

In summary, the N-end rule pathway has been shown to be an ancient pathway that likely pre-dates the advent of the UPS in eukaryotes [12,38,40]. Furthermore, its hierarchical organization and some of its enzymatic components have been conserved throughout evolution.

Plant N-recognins: a continuing story?
PRT1, the first N-recognin identified in plants, was isolated by Bachmair and colleagues [20,41] in an elegant genetic screen that was based on the mutagenesis of transgenic plants expressing an N-end rule reporter (Figure 1) that contained an N-terminal Ub, the junctional residue Phe, and dihydrofolate reductase (DHFR) at the C-terminus. These plants were grown in the presence of the drug methotrexate at a concentration that efficiently inhibited endogenous DHFR, resulting in plant death. However, mutation of the N-recognin PRT1 led to the accumulation of the transgenic Phe-DHFR protein, resulting in resistance to methotrexate [41]. PRT1 was later shown to bind substrates bearing aromatic hydrophobic residues (Phe, Trp and Tyr) at their N-terminus, but not proteins starting with aliphatic hydrophobic residues such as Leu and Ile [18].

PRT6, the second known plant N-recognin, was identified based on its sequence similarity to yeast UBR1 [17]. Although PRT6 contains a UBR domain, it does not have the so-called N-domain, which in UBR1 is required for the recognition of substrates with hydrophobic destabilizing residues [17,36]. In agreement with its domain architecture, PRT6 was found to bind to N-end rule substrates with the basic N-terminal residue Arg, but not to proteins starting with the hydrophobic residues Leu and Phe [17].

Despite the progress made in the characterization of plant N-recognins, the known specificities of PRT1 and PRT6 do not account for all primary destabilizing residues identified in plants. For example, Leu and Ile were shown to exert a destabilizing effect on N-end rule reporter proteins [19], but substrates with these N-terminal residues are not recognized by either of the two known plant N-recognins. This strongly suggests that additional N-recognins remain to be discovered.

In addition to PRT6, several *Arabidopsis* proteins show sequence similarities to known N-recognins. For instance, BLASTP searches using the 570-kDa N-recognin UBR4 from mouse as a query, retrieves a protein of similar size in *Arabidopsis*, which is known as TIR3 (TRANSPORT INHIBITOR RESPONSE3), DOC1 (DARK OVEREXPRESSION OF CAB1) or BIG (referring to its large size). Mutant alleles of *BIG* were isolated in multiple independent genetic screens [42–46], suggesting that BIG might be involved in the regulation or crosstalk of various signal transduction pathways [43]. The sequence similarities between BIG and UBR4 suggest that BIG could function as an N-recognin and bind N-end rule substrates with both basic and bulky hydrophobic primary destabilizing residues. However, given the enormous size of BIG, it is possible that this protein has numerous functional domains, some of which could be independent of its putative role as an N-recognin.

Another candidate for a novel plant N-recognin is *Arabidopsis* ClpT [47,48]. This protein bears sequence similarities to the bacterial N-recognin ClpS [34,35,37,47] and is predicted to localize to the chloroplast [47]. The presence of ClpT [47] and a Clp-like protease in chloroplasts [49] raises the exciting possibility that this organelle might contain a Ub-independent (i.e. prokaryotic) N-end rule pathway.

Finally, it is possible that the genomes of plants encode N-recognins that have, like PRT1, no sequence similarities to other known N-recognins. For the discovery of such plant-specific N-recognins, sophisticated genetic screens [41] or affinity purification assays using artificial N-end rule substrates [24] might be required.

Functions of the N-end rule pathway in plants
The N-end rule pathway has been shown to play essential roles in a multitude of cellular and developmental processes in fungi and animals. These include heme sensing [50], peptide import [51–53], chromosome segregation [54], DNA repair [55] and apoptosis [56,57], as well as the regulation of meiosis, neurogenesis, cardiovascular development and pancreatic functions [12,58–64].

Functions of the N-end rule pathway in plants have begun to emerge only recently, but are now rapidly amassing (Figure 3). Initial evidence for the involvement of the N-end rule pathway in controlling plant growth and development stemmed from a genetic screen for mutants with abnormal leaf senescence. Satoko Yoshida *et al.* isolated the *delayed leaf senescence 1* mutant in *Arabidopsis*, which is affected in the R-transferase AtATE1 [21]. They further showed that the *Arabidopsis* genome encodes a second, closely related R-transferase, AtATE2. It was later demonstrated that the two *Arabidopsis* R-transferases have almost identical expression patterns [65] and act in a functionally redundant manner [65,66].

An additional function of the plant N-end rule pathway was uncovered by Tara J. Holman *et al.* [66] who identified a mutant allele of the N-recognin PRT6 in a genetic screen aimed at identifying genes that control seed ripening and germination. The decreased germination potential observed in *prt6* mutants was found to correlate with a hypersensitive response to exogenously added abscisic acid (ABA), a known inhibitor of germination [67]. This result suggested that PRT6 might be involved in the removal of
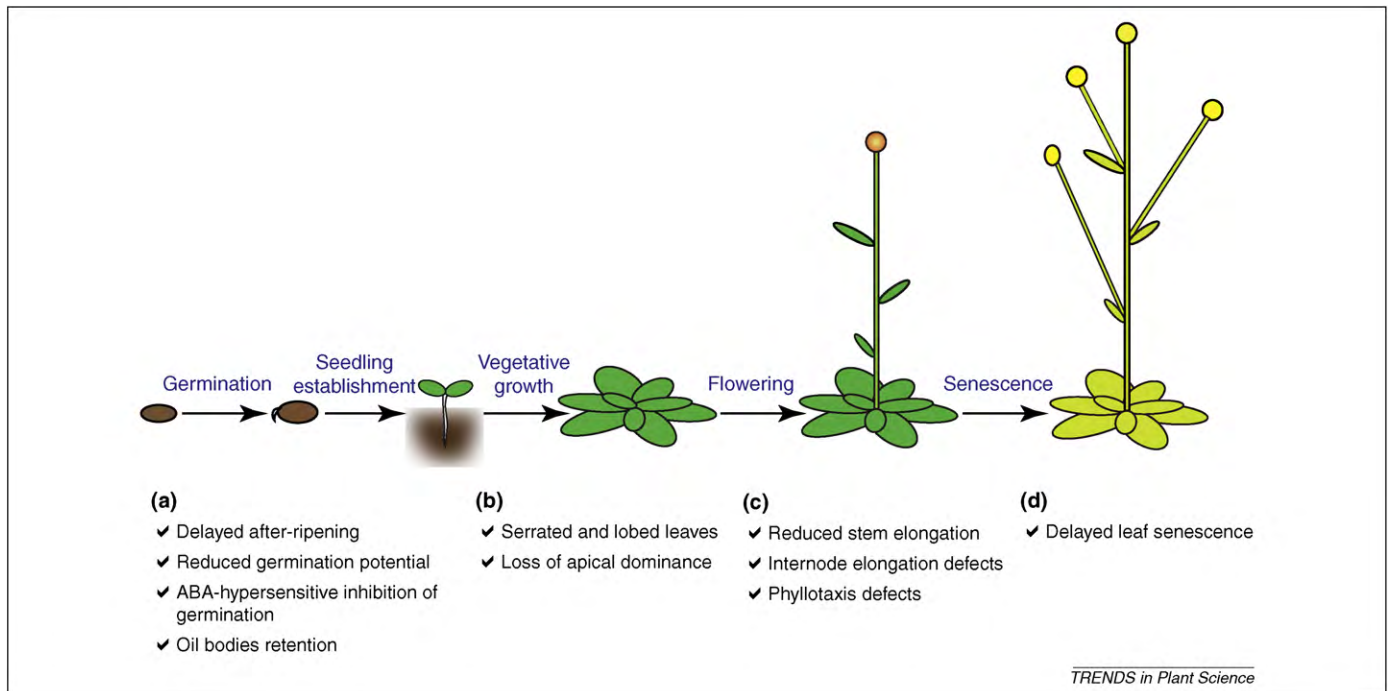
Figure 3. Functions of the N-end rule pathway in *Arabidopsis*. The N-end rule pathway is involved in the regulation of multiple developmental processes throughout the life cycle of the plant, from seed maturation and germination to senescence. Phenotypic abnormalities observed in mutant plants affected in components of the N-end rule pathway (as indicated) are described. **(a)** *prt6* and *ate1 ate2* mutants [66]. **(b)** and **(c)** *prt6* and *ate1 ate2* mutants [65]. **(d)** *dls1* and *ate1 ate2* mutants [21,65].

ABA sensitivity prior to germination. Genetic interaction analyses with components of the ABA signaling pathway further implied that PRT6 functions upstream of the ABA response regulator ABA INSENSITIVE 3 (ABI3) in the control of germination [66]. Notably, plants mutant for both *Arabidopsis* R-transferases (i.e. *ate1 ate2*) exhibit a similar phenotype to that of *prt6* mutants, strongly suggesting that the N-end rule substrates responsible for the germination defects are first arginylated by R-transferases before they are processed by PRT6 [66].

In addition to controlling leaf senescence and seed germination, the N-end rule pathway is involved in regulating shoot and leaf development. *ate1 ate2* double-mutant plants, as well as *prt6* single mutants, exhibit a wide range of phenotypic abnormalities, including serrated and lobed leaves, loss of apical dominance, and stem and internode elongation defects [65]. A detailed characterization of the leaf morphology defects of *ate1 ate2* plants revealed that the key regulator of leaf development *BRE-VIPEDICELLUS* (*BP*) [68] is mis-expressed in *ate1 ate2* plants [65]. *BP* expression is normally excluded from *Arabidopsis* leaves through activities of the ASYMMETRIC LEAVES 1 (AS1) transcription factor complex [69] and of the phytohormone auxin [70]. The results of genetic analyses suggest that the N-end rule pathway acts redundantly with AS1, but independently of auxin in controlling leaf development [65].

All functions of the plant N-end rule pathway described above are mediated by its arginylation branch, which targets only a subset of all possible substrates for degradation (Figure 2). It is thus likely that additional functions of the N-end rule pathway that do not depend on arginylation remain to be discovered and that its roles in plants will ultimately turn out to be as diverse as in animals and fungi.

N-end rule substrates: searching for needles in a haystack

Although the N-end rule pathway has been examined in different model organisms and was found to regulate multiple cellular and developmental processes [12,60,71,72], our understanding of the molecular mechanisms underlying its functions is currently limited, mainly because only a few of its substrates have been discovered. In the following paragraph, we will outline how N-end rule substrates are generated and why their systematic identification poses a formidable challenge.

Although most studies of the N-end rule pathway were carried out using artificial N-end rule substrates generated through the Ub fusion technique [73] (Figure 1), endogenous proteins are typically synthesized with an N-terminal Met (a stabilizing residue), which should preclude degradation by the N-end rule pathway. However, the N-terminal Met can be removed from proteins by methionine aminopeptidases (MetAPs). This cleavage occurs only if the second residue (which would then be exposed) has a relatively small side chain [74–76]. Although most of the residues that enable MetAPs to cleave off Met are stabilizing (e.g. Gly and Ala), the removal of Met in front of Cys results in a protein with a destabilizing N-terminal residue (Figure 1). Examples for the generation of N-end rule substrates through this mechanism are the mouse proteins RGS4 (Regulator of G-protein Signaling 4), RGS5 and RGS16, whose newly exposed N-terminal Cys residue is oxidized prior to arginylation and degradation [30,31]. There are more than 200 *Arabidopsis* proteins with an

amino acid sequence that begins with Met–Cys. However, because a destabilizing residue is only one requirement for a functional N-degron (see above), N-end rule substrates cannot be identified based on the presence of a Met–Cys pair at the N-terminus alone. In yeast, another exception to the formation of N-end rule substrates is proteins whose N-terminal residue is acetylated (Figure 1). These include proteins whose N-terminal Met can be acetylated or proteins cleaved by MetAPs [32]. Several substrates targeted for degradation through this recently uncovered branch of the N-end rule pathway have been identified, including the mating type transcription factor MATα2 [32].

Other known N-end rule pathway substrates, such as the cohesin subunit SCC1 (Sister Chromatid Cohesion protein 1) in yeast [54], are generated after cleavage by a specific protease, resulting in the release of protein fragments bearing destabilizing N-terminal residues (Figure 1). It is assumed that many substrates of the N-end rule pathway are generated after such proteolytic cleavage [54,56,57]. Unfortunately, the requirement for such processing precludes the systematic identification of N-end rule substrates using, for example, bioinformatics approaches.

In plants, no *bona fide* N-end rule substrates have been identified to date. One promising candidate is the *Arabidopsis* RPM1 INTERACTING PROTEIN 4 (RIN4) protein, which is involved in the response to pathogens and is cleaved by the bacterial protease AvrRpt2 upon infection by *Pseudomonas syringae* [77–80]. Cleavage of RIN4 by AvrRpt2 occurs at two sites, releasing short-lived protein fragments with the destabilizing residues Asn and Asp, respectively, in N-terminal position [81]. Based on the expression of truncated versions of RIN4, which were fused to green fluorescent protein, it has been proposed that the RIN4 protein fragments might be degraded through the N-end rule pathway [82]. However, further experiments are required to test this conjecture and to confirm an involvement of the plant N-end rule pathway in pathogen response.

Conclusion

Despite the rapid progress made in recent years in characterizing the plant N-end rule pathway and in identifying its physiological functions, many open questions remain. Key aims for the years to come will be identification of the complete set of plant N-recognins and the discovery of the first *bona fide* N-end rule substrates in plants. This information should not only extend our knowledge of the functions of the plant N-end rule pathway, but would also provide insights into the underlying molecular mechanisms.

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