From start to finish: amino-terminal protein modifications as degradation signals in plants

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Summary

The amino- (N-) terminus (Nt) of a protein can undergo a diverse array of co- and post-translational modifications. Many of these create degradation signals (N-degrons) that mediate protein destruction via the N-end rule pathway of ubiquitin-mediated proteolysis. In plants, the N-end rule pathway has emerged as a major system for regulated control of protein stability. Nt-arginylation-dependent degradation regulates multiple growth, development and stress responses, and recently identified functions of Nt-acetylation can also be linked to effects on the *in vivo* half-lives of Nt-acetylated proteins. There is also increasing evidence that N-termini could act as important protein stability determinants in plastids. Here we review recent advances in our understanding of the relationship between the nature of protein N-termini, Nt-processing events and proteolysis in plants.

1. Introduction

The amino- (N-) terminus (Nt) is a positional feature common to all proteins, and has a number of characteristics that provide unique biochemical and structural properties to the associated polypeptide. Proteins are created with a methionine (Met; or formyl-methionine, fMet) at their N-terminus; however N-termini can subsequently undergo a wide range of modifications and/or processing events (Giglione *et al.*, 2015; Varland *et al.*, 2015). In a majority of proteins, Nt-Met is co-translationally cleaved by METHIONINE AMINO-PEPTIDASES (MetAPs), exposing novel Nt-residues (Giglione *et al.*, 2003). Furthermore, many proteins are synthesised with Nt-transit peptides, excised post-translationally once a protein is delivered to its subcellular destination (van Wijk, 2015). Enzymatic modification of Nt-residues is also common, including acetylation and myristolyation of α-amino groups, oxidation of cysteine (Cys) thiols, deamidation of asparagine (Asn) and glutamine (Gln), and many other modifications (Gibbs *et al.*, 2014a; Giglione *et al.*, 2015). Moreover, Nt-conjugations, such as arginylation and ubiquitination can also occur (Gibbs *et al.*, 2014a; Varland *et al.*, 2015). Therefore, Nt-residues have emerged as key regulatory loci in proteins that can significantly impact protein activity.

One major function for protein N-termini is in determining the in vivo half-lives of corresponding proteins via the N-end rule pathway of protein degradation, a set of ancient proteolytic systems present in prokaryotes and eukaryotes (Bachmair *et al.*, 1986; Varshavsky, 2011; Gibbs *et al.*, 2014a). In the latter, the N-end rule pathway has been co-opted to the ubiquitin proteasome system, targeting proteins for destruction by the 26S proteasome through conjugation of a polyubiquitin chain (Gibbs *et al.*, 2014a). The N-end rule

relates the in vivo half-life of a protein to the nature of its Nt-residue, which alongside other requisite features (an unstructured, exposed N-terminus and accessible downstream lysine(s)) form a degradation signal called the N-degron (Fig. 1). N-degrons are typically conditional, being exposed and subsequently recognised by ubiquitin E3 ligases (N-recognins) only under certain situations or in response to specific signals. Consequently, protein destruction via the N-end rule pathway has important roles in signal perception and transduction, as well as general proteostasis and protein quality control. Two divisions of the N-end rule pathway have been discovered – the arginylation (Arg/) N-end rule, which recognises substrates with unmodified basic or hydrophobic residues, and the acetylation (Ac/) N-end rule, which targets proteins bearing certain Nt-acetylated residues (Bachmair *et al.*, 1986; Hwang *et al.*, 2010; Varshavsky, 2011; Gibbs *et al.*, 2014a; Lee *et al.*, 2016). In this review we discuss recent advances in our understanding of these pathways, their protein targets and their wide ranging functions in plants.

2. The plant Arg/N-end rule: a central regulator of development and stress signalling

In plants, there are two confirmed N-recognins of the Arg/N-end rule: PROTEOLYSIS1 (PRT1) and PRT6, which bind to substrates bearing aromatic or basic Nt residues, respectively (Potuschak *et al.*, 1998; Garzon *et al.*, 2007). This is in contrast to the Arg/N-recognins of yeast and mammals, which are able to recognise both classes of destabilising residue via separate binding domains within the same polypeptide (Varshavsky, 2011; Gibbs *et al.*, 2014a). Although the Nt-targets of PRT1 have been characterised using artificial reporter proteins, natural substrates and biological functions for this N-recognin remain elusive (Gibbs *et al.*, 2014a). In contrast, the PRT6-mediated division of the plant Arg/N-end rule has emerged as an important regulator of growth, development and stress-associated responses (Fig. 2). PRT6 recognises substrates bearing Nt-Arg (Garzon *et al.*, 2007), which can be exposed by peptidases, or arise as a result of successive Nt-processing events. For example, Nt-aspartate (Asp) and Nt-glutamate (Glu) can be arginylated by ARGINYL tRNA TRANSFERASES (ATE) to produce a primary N-degron, whilst Nt-Asn and Nt-Gln can be deamidated by NTAN1 to NTAQ1 enzymes prior to arginylation (Graciet *et al.*, 2010; Gibbs *et al.*, 2014a). Furthermore, Nt-Cys can be arginylated in an oxidation-dependent manner (see below).

Diverse functions for the Arg/N-end rule have been uncovered in *Arabidopsis* through analysis of mutants of the pathway that accumulate endogenous substrates. Key developmental roles include the regulation of seed dormancy and germination, seedling development and establishment, leaf and shoot development, and the control of leaf

senescence (Fig. 2) (Yoshida *et al.*, 2002; Graciet *et al.*, 2009; Holman *et al.*, 2009; Abbas *et al.*, 2015). The pathway mediates low-oxygen (hypoxia) and nitric oxide (NO) sensing in plants as well as animals (Hu *et al.*, 2005; Lee *et al.*, 2005; Gibbs *et al.*, 2011; Gibbs *et al.*, 2014b), and acts at the interface of abscisic acid (ABA), gibberellin and ethylene signalling during stress and development (Gibbs *et al.*, 2011; Licausi *et al.*, 2011; Gibbs *et al.*, 2014b; Marin-de la Rosa *et al.*, 2014; Gibbs *et al.*, 2015; Mendiondo *et al.*, 2015). Recently, the pathway was also linked to the plant immune response (de Marchi *et al.*, 2016). The Arg/N-end rule pathway has also been investigated in the moss *Physcomitrella patens*, an early-evolving land plant, where an ATE loss-of-function mutant was shown to be defective in gametophytic development (Schuessele *et al.*, 2016). Furthermore, the pathway has been shown to control developmental and stress responses in barley, a monocotyledonous crop species (Mendiondo *et al.*, 2015).

Despite this wide range of functions for the Arg/N-end rule, only one group of substrates has been identified: The group VII ETHYLENE RESPONSE FACTOR (ERFVII) transcription factors, characterised by a highly conserved Nt-motif initiating with the residues Nt-Met-Cys (Gibbs *et al.*, 2011; Licausi *et al.*, 2011). Nt-processing of ERFVIIs, catalysing their degradation, occurs in several steps (Fig. 3a): Nt-Met is removed by MetAPs to reveal Nt-Cys, which can be oxidised by plant cysteine oxidases (PCOs), using oxygen as a cofactor (Weits *et al.*, 2014). Oxidised Nt-Cys is then proposed to be arginylated by ATEs, followed by PRT6-dependent ubiquitination (Gibbs *et al.*, 2011; Licausi *et al.*, 2011). NO is also required for this degradation (Gibbs *et al.*, 2014b). Oxygen- and NO-dependant destruction of ERFVIIs therefore acts as a signal-responsive “switch” determining their half-life. Consequently, ERFVIIs play a central role in the coordination of transcriptional responses to both of these gaseous molecules, which function as important metabolic, developmental and stress-associated signals in plants (Gibbs *et al.*, 2015).

Arg/N-end rule mutant phenotypes are highly pleiotropic, indicating there may be other protein targets of the pathway. Arabidopsis contains more than 200 proteins initiating Nt-Met-Cys, and it is possible that the stability of a cohort of these could be controlled by Nt-Cys oxidation similarly to the ERFVIIs (Gibbs *et al.*, 2014a). It was previously reported that RPM1-INTERACTING PROTEIN 4 (RIN4), a component of the plant immune response, may become a proteolytic target following cleavage by *Pseudomonas syringae* effector cysteine protease *AvrRpt2*, which reveals Nt-Asn and -Asp (Takemoto & Jones, 2005), although direct genetic or biochemical evidence for this is still lacking. In yeast and animals, the pathway counteracts apoptosis through degrading pro-apoptotic peptide fragments, and similar functions may be present in plants, where METACASPASE9 activity generates many protein fragments bearing destabilising residues (Tsiatsiani *et al.*, 2013; Gibbs *et al.*, 2014a). Large scale proteomics

studies are now being employed to identify and confirm novel targets of the Arg/N-end rule, by looking at quantitative differential protein accumulation in *prt6* and *ate* mutants (Zhang *et al.*, 2015), or by 'fishing' for N-end rule enzyme interaction-partners (Hoernstein *et al.*, 2016). The continual improvement of N-terminomic methods will also help with this endeavour (Venne *et al.*, 2015).

3. Nt-acetylation as a putative degradation signal in plants

During protein synthesis, the α-amino group of Nt-residues can be co-translationally acetylated by ribosome-associated Nt-acetyltransferases (NATs) (Giglione *et al.*, 2015; Varland *et al.*, 2015). This either occurs directly on Nt-Met, or on the second residue following Met-removal by MetAP. Three NATs (NATA, B, and C) catalyse the majority of these modifications, with each having distinct substrate specificities. Post-translational Nt-acetylation also likely occurs (Giglione *et al*., 2015; Bienvenut *et al*., 2011). Nt-acetylation is highly prevalent in the proteomes of eukaryotes, but its functions are not well characterised. In plants, NAT loss-of-function mutants have been linked to growth defects and reduced photosynthetic efficiency (Gibbs, 2015). It has also been shown that drought-induced increases in ABA trigger a reduction in NATA levels that leads to reduced global Nt-acetylation and improved tolerance to water-deficit (Linster *et al.*, 2015).

In 2010 it was demonstrated in yeast that Nt-acetylation of proteins can act as a signal for degradation, as part of the Ac/N-end rule pathway (Fig. 3b) (Hwang *et al.*, 2010). Two E3 ligases that recognise Nt-acetylated (Ac/) N-degrons were identified: the ER-associated DOA10/TEB4 and cytosolic NOT4 (Lee *et al.*, 2016). Ac/N-degrons were shown to be conditional, only becoming accessible in misfolded proteins or proteins not bound to interaction partners (Shemorry *et al.*, 2013; Lee *et al.*, 2016). This pathway has recently been linked to important functions in human health, with naturally occurring Nt-variants of REGULATOR OF G PROTEIN SIGNALLING (RGS) proteins increasing susceptibility to hypertension due to altered rates of degradation via their differentially acetylated N-termini (Park *et al.*, 2015). A functional Ac/N-end rule pathway has not yet been identified in plants, although NATs, and proteins with high sequence similarity to both DOA10 and NOT4, exist in *Arabidopsis* (Gibbs *et al.*, 2014a; Gibbs, 2015). Interestingly, mutants of the *Arabidopsis* DOA10-like gene *ECERIFERUM9/SUPPRESSOR OF DRY2 DEFECTS1* (*CER9/SUD1*) display ABA-hypersensitivity during seed germination, similar to the ABA-associated phenotypes observed in NATA-deficient plants (Zhao *et al.*, 2014; Linster *et al.*, 2015). If Nt-acetylation acts as a degradation signal, accumulation of its substrates would be expected in both the *natA* and *cer9*/*sud1* mutants; it is therefore possible that proteins associated with ABA signalling might be targets of a plant Ac/N-end rule pathway.

More direct evidence for an association between Nt-acetylation and protein stability in plants has recently been uncovered in *Arabidopsis*. It was shown that SUPPRESSOR OF NPR1, CONSTITUTIVE1 (SNC1), a key regulator of plant immunity, accumulates in *natA* mutants leading to increased pathogen tolerance (Xu *et al.*, 2015). This suggests that Nt-acetylation of SNC1 by NATA might create a functional Ac/N-degron in this protein. Interestingly, SNC1 was shown to occur in two Nt-isoforms; the second variant is Nt-acetylated by NATB, which appears to *stabilise* the protein (Xu *et al.*, 2015). This contrasting, variant-specific consequence of NAT activity suggests that the effects of Nt-acetylation of protein half-life are highly complex. One possible explanation, as previously postulated for Ac/N-end rule substrates in yeast and mammals (Shemorry *et al.*, 2013; Park *et al.*, 2015), is that stabilization of the NATB-modified SNC1 variant may stem from the ability of a longer-lived Nt-acetylated version of SNC1 to form a less rapidly dissociating protective complex with its cognate ligands *in vivo*, in contrast to an analogous but more rapidly dissociating complex that involves the NATA-modified (short-lived) version. It will now be important to further unravel the influence of Nt-acetylation on protein half-life and determine whether plant DOA10 or NOT4-like ubiquitin E3 ligases represent functional components of a plant Ac/N-end rule pathway.

4. The N-terminus as a stability determinant in plastids

The chloroplast proteome comprises proteins of nuclear origin as well as those encoded by the organellar genome (van Wijk, 2015). The N-termini of proteins from these different sources undergo a range of processing events that collectively control the diversity of the mature chloroplast N-terminome (Fig. 3c). Surprisingly, a large number of chloroplastic proteins are represented by multiple Nt-proteoforms, suggesting that processing of N-termini is complex, dynamic and that different Nt-variants may have different functions (Rowland *et al.*, 2015). Nuclear encoded proteins make up more than 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 cleaved by the stromal processing peptidase (SPP) to reveal new Nt-amino acids, which can then be further modulated by one of at least seven amino-peptidases (van Wijk, 2015). SPP cleaves at a range of different sites, and at single or multiple positions; this enzymatic promiscuity coupled with subsequent amino-peptidase activity has been proposed to ensure that unfavourable (potentially destabilising) Nt-residues are removed (Rowland *et al.*, 2015; van Wijk, 2015). In contrast to nuclear-derived proteins, plastid-encoded proteins initiate with Nt-fMet, and undergo co-translational deformylation followed by Nt-Met excision, which are both essential for normal plastid development (Giglione *et al.*, 2015; van Wijk, 2015). Interestingly Met-retention on chloroplast proteins has previously been linked

to protein instability (Giglione *et al.*, 2003), whilst fMet can act as a destabilising residue in bacteria, and possibly also chloroplasts (Piatkov *et al.*, 2015). Co-translational and post-translational Nt-acetylation also occurs on chloroplastic proteins, which appears to enhance protein stability (Bienvenut *et al.*, 2011); recently a nuclear encoded chloroplast-targeted NAT that likely catalyses this modification has been identified (Dinh *et al.*, 2015).

Accumulating evidence points towards a relationship between N-termini and protein stability in plastids. Using artificial protein-GFP fusions in transplastomic tobacco it was shown that the identity of the penultimate Nt-residue strongly correlates with differences in protein accumulation (Apel *et al.*, 2010). Some residues led to protein stabilisation, whilst others (unrelated to the prokaryotic N-end rule; see below) reduced abundance considerably. It has also been reported that labile recombinant proteins produced in plastids can be stabilised by Nt-translational fusions (Lenzi *et al.*, 2008; Apel *et al.*, 2010).

Due to the cyanobacterial origin of chloroplasts, it is possible that a *bona fide* plastid N-end rule pathway could be similar to that in prokaryotes, which differs to that found in eukaryotes (Mogk *et al.*, 2007; van Wijk, 2015). in *Escherichia coli*, primary destabilising Leucine (Leu) and Phenylalanine (Phe) residues can be conjugated to proteins bearing Nt-Arginine (Arg) or –Lysine (Lys) via leucyl/pheylalanyl(Leu/Phe)-tRNA protein transferase, or in other prokaryotes by transferases with different specificities (Graciet *et al.*, 2006). Substrate selection is mediated by the caseinolytic protease (Clp) S protein (ClpS), which delivers N-degron-bearing substrates to the ClpAP protease for destruction (Mogk *et al.*, 2007). No Leu/Phe-transferase-like sequences are present in the chloroplast genome, though ClpS-(called ClpS1) and ClpAP-like proteins, encoded in the nucleus, accumulate in chloroplasts (Nishimura *et al.*, 2013). Recently a novel Clp protein unique to photosynthetic eukaryotes, ClpF, has also been identified. ClpF is proposed to act as a binary adaptor alongside ClpS1 for selective substrate recognition and delivery to the Clp protease, suggesting evolutionary adaptation of the chloroplast Clp system (Nishimura *et al.*, 2015). Affinity experiments using recombinant ClpS1 identified a number of stromal binding partners that also had increased abundance in *clps1* mutants; these interactions were abolished when conserved residues in the putative N-degron binding pocket of ClpS1 were mutated (Nishimura *et al.*, 2013). Moreover, the Nt-domains of these targets share some features with confirmed substrates of the *E. coli* ClpS, and one of these proteins, Glutamyl-tRNA reductase (GluTR), directly interacts with ClpS1 via its N-terminus (Nishimura *et al.*, 2013; Apitz *et al.*, 2016). The GluTR N-terminus also interacts with membrane bound GluTR binding protein (GBP), which stabilises GluTR, suggesting that a putative N-degron shielding effect similar to that which occurs in the Ac/N-end rule pathway may also exist in plastids. Based on these varied observations, it seems likely that N-termini dictate protein stability in chloroplasts, possibly via

a modified variant of the prokaryotic N-end rule pathway; the exact mechanisms involved now need to be established.

5. Concluding remarks

Here we have briefly reviewed current knowledge on the diversity of plant Nt-modifications and their influence on protein stability. It is interesting to note that N-degrons represent one of the earliest evolving determinants of protein instability, due to their presence in both prokaryotic and eukaryotic kingdoms, and therefore are likely to play important roles during many more aspects of plant life than is currently appreciated. The challenge is now to further define the enzymes and rules coordinating regulated destruction via the various N-end rule pathways in plants, and to identify protein substrates and physiological processes dependent on this regulation.

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Figure legends

Figure 1. Features of N-degrons. Diagrammatic representation of an N-end rule substrate being polyubiquitinated (Ub) by its respective E2 and E3 (N-recognin) ubiquitin ligase, highlighting the three key features that determine an N-degron: (1) A primary N-terminal destabilising amino acid (which may be either unmodified or acetylated); (2) An unstructured N-terminal region ensuring the Nt-residue is exposed and accessible; (3) An appropriately positioned downstream lysine(s) to act as a receptor site for ubiquitin conjugation.

Figure 2. Functions for the N-end rule pathway in plant development and stress response. The Arg/N-end rule pathway controls a wide range of processes in *Arabidopsis*, including seed germination, photomorphogenesis, submergence response, shoot and leaf development, stomatal aperture, leaf senescence and pathogen responses. For each of these processes the N-end rule enzymes (blue), substrates (blue, underlined) and gaseous signals (orange) involved are shown. The Ac/N-end rule is still not confirmed in plants, but links between NATs (red) and SNC1 (red, underlined) stability during the response to pathogen attack have been reported, suggesting that the pathway may exist and function during biotic stress. Arrows and bars represent positive and negative influences, respectively. PRT6, PROTEOLYSIS6; ATE, ARGINYL tRNA-TRANSFERASE; ERFVII, group VII ERF

transcription factors; O_2, oxygen; NO, nitric oxide; NATA/B, N-TERMINAL ACETYLTRANSFERASE A/B; SNC1, SUPPRESSOR OF NPR1, CONSTITUTIVE 1.

Figure 3. Diversity of N-terminal processing events and their influence on protein stability. (a) Control of Met-Cys-initiating proteins (e.g. ERFVII transcription factors in this example) via the Cys branch of the Arg/N-end rule pathway. Nt-Met (M) is cleaved by METHIONINE AMINO PEPTIDASES (MetAP); Nt-Cys oxidation *in vivo* requires both oxygen and nitric oxide, and may be catalysed by PLANT CYSTEINE OXIDASE (PCO) enzymes. Oxidised Nt-Cys (C*) is then proposed to be arginylated by ARGINYL tRNA-TRANSFERASES (ATE); Nt-Arg (R), as a destabilising residue, is then likely bound by the ubiquitin E3-ligase/N-recognin PROTEOLYSIS6 (PRT6), and degraded via the 26S proteasome. The Nt-arginylation and PRT6-recognition steps are both supported by the accumulation of ERFVIIs and artificial reporter proteins in *ate1ate2* and *prt6* mutants, respectively (Gibbs *et al.*, 2011; Licausi *et al.*, 2011; Gibbs *et al.*, 2014b). **(b)** The Ac/N-end rule pathway (confirmed in yeast and mammals; putative in plants). Nt-Met can be acetylated (Ac) by N-TERMINAL ACETYLTRANSFEREASES (NATs) if the penultimate Nt-amino acid is bulky and hydrophobic (Φ). Alternatively, Nt-Met may first be cleaved by MetAP and the newly exposed Nt-residue (X) acetylated. Ac/N-degrons are recognised and targeted for proteasomal degradation in yeast and mammals by one of two E3s/N-recognins; DOA10/TEB4 or NOT4. Proteins with high similarity to these N-recognins are present in plants. **(c)** N-terminal processing in chloroplasts and putative effects on protein stability (X and Z represent any amino acid). Chloroplast-genome-derived proteins are deformylated by PROTEIN DEFORMYLASES (PDF), and then may be processed further by MetAPs, one of several other plastid aminopeptidases (APs), and/or NATs. Nuclear derived proteins first have their chloroplast transit peptide (cTP) cleaved by STROMAL PROCESSING PROTEASE (SPP), and then may be subjected to further processing by APs or NATs. Putative effects of these Nt-modifications on protein stability are shown.