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Conditional Protein Function via N-Degron Pathway–Mediated Proteostasis in Stress Physiology

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proteolysis, protein degradation, proteases, adaptation, acclimation, ubiquitin, proteasome, N-end rule pathway

Abstract

The N-degron pathway, formerly the N-end rule pathway, regulates functions of regulatory proteins. It impacts protein half-life and therefore directs the actual presence of target proteins in the cell. The current concept holds that the N-degron pathway depends on the identity of the amino (N)-terminal amino acid and many other factors, such as the follow-up sequence at the N terminus, conformation, flexibility, and protein localization. It is evolutionarily conserved throughout the kingdoms. One possible entry point for substrates of the N-degron pathway is oxidation of N-terminal Cys residues. Oxidation of N-terminal Cys is decisive for further enzymatic modification of various neo–N termini by arginylation that generates potentially neofunctionalized or instable proteoforms. Here, I focus on the posttranslational modifications that are encompassed by protein degradation via the Cys/Arg branch of the N-degron pathway—part of the PROTEOLYSIS 6 (PRT6)/N-degron pathway—as well as the underlying physiological principles of this branch and its biological significance in stress response.

Contents

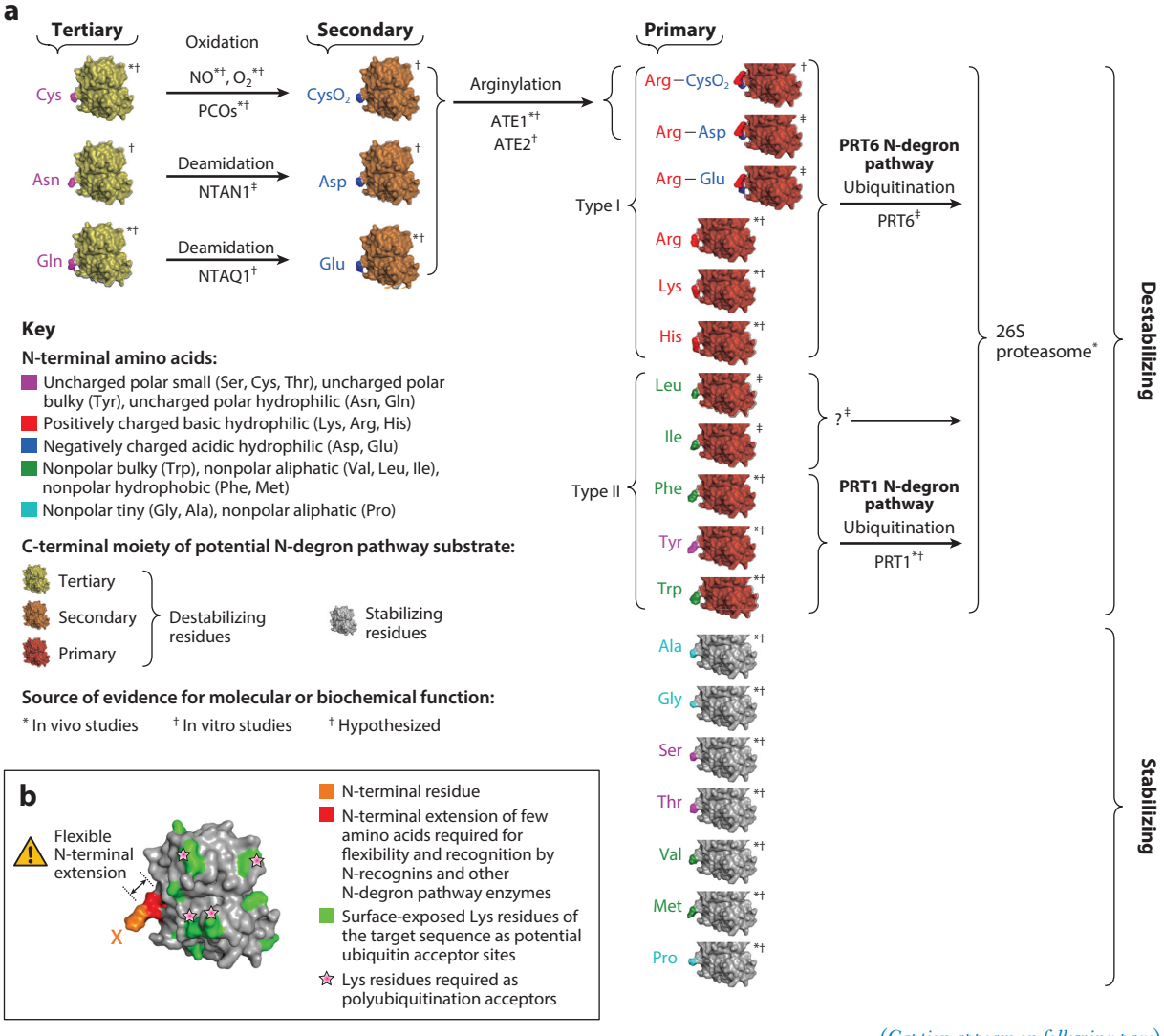
N-degron: neo-N-terminal residue(s) that gets recognized by the E3 ubiquitin (Ub) ligases of the N-degron pathway (N-recognins)

INTRODUCTION

In this review, I focus on posttranslational modifications (PTMs) via the Cys/Arg branch of the N-degron pathway (or, Cys/Arg branch), the underlying physiological principles of this branch, and its biological significance in stress response. The N-degron pathway, formerly the "N-end rule pathway," is currently undergoing a rebranding (89, 121); it comprises diverse enzyme classes, such

as peptidases, amidases, oxygenases, transferases, and ubiquitin (Ub) ligases, and the proteasome, which were suggested to be involved in specific PTMs of either protein N termini or entire protein substrates following modification via this branch and other related branches of the pathway. In many cases, these assumptions relied on indirect effects, such as the degradation of proteins bearing so-called destabilizing residues at their N termini. Taken together, these enzymes have the potential to act on protein substrates in a series of biochemical events (**Figure 1**), but not all of these steps will necessarily happen nor—as is most critical in this context—can they be detected in vitro or even in vivo. Therefore, in this review, I discuss biochemical, physiological, and genetic data that highlight representatives of the enzyme classes that are involved in the N-degron pathway. In particular, I focus on PLANT CYSTEINE OXIDASES (PCOs), ARGINYL-TRANSFER RNA (tRNA):PROTEIN ARGINYLTRANSFERASES (ATEs) (also known as arginine transfer enzymes), and PROTEOLYSIS 6 (PRT6)—the last is the bona fide E3 Ub protein ligase in

Degron: protein degradation signal within a protein sequence that makes it short-lived in vivo or in vitro; can be dependent on posttranslational modifications such as cleavage or amino acid derivatization



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Contemporary understanding of the plant N-degron pathway. The N-degron pathway describes the relation of the N-terminal amino acid residue and the metabolic stability of a protein. (*a*) The classical N-degron pathway, previously known as the so-called Arg/N-end rule pathway in yeast and mammals, is split into various subbranches and built up as a biochemical reaction cascade. In plants, it is subdivided into the PROTEOLYSIS 6 (PRT6)/N-degron pathway and the PRT1 N-degron pathway, addressing type I (Arg, Lys, His) and type II (Phe, Tyr, Trp, Leu, Ile) residues, respectively. Protein N termini can be classified into stabilizing and destabilizing according to the first amino acid. There are three groups of destabilizing residues: primary, secondary, or tertiary. Substrates bearing an N-terminal primary destabilizing residue, such as an Arg or Phe, can be directly recognized by two different E3 ubiquitin (Ub) ligases (the so-called N-recognins, PRT1 and PRT6). In the PRT6 N-degron pathway, the secondary destabilizing residues Asp, Glu, and dioxygenated Cys ($CysO_2$) can be enzymatically formed by N-terminal amidases (NTAs) from Asn and Gln and by Cys dioxygenases (PLANT CYSTEINE OXIDASEs, PCOs) from Cys and molecular oxygen (O_2). Cys can also be oxidized nonenzymatically via NO and reactive oxygen species (ROS). Arginyltransferases (ATEs, or arginine transfer enzymes) can attach an N-terminal Arg to secondary destabilizing residues to generate a potentially highly instable primary destabilizing Arg N terminus. In plants, two ATE homologs and individual putative orthologs of mouse Asn- and Gln-specific NTAs (NTAN1 and NTAQ1, respectively) were found. In the PRT1 N-degron pathway, according to current knowledge, no further modification of the N-terminal end is required to be potentially recognized by the E3 Ub ligase PRT1. The predicted specificities of the two bona fide plant N-recognins PRT1 and PRT6 are shown. The two known *Arabidopsis* N-recognins were identified by their function (PRT1, 46 kDa) and by homology to the UBR-box from *Saccharomyces cerevisiae* UBR1p (PRT6, 224 kDa). Superscript symbols refer to the molecular biochemical function. The asterisk (*) indicates evidence from in vivo studies exists, the dagger (†) indicates evidence from in vitro studies exists, and the double dagger (‡) indicates hypotheses that have yet to be tested. (*b*) N-degrons comprise as main determinants neo-N-terminal residues that get recognized by the E3 Ub ligases of the N-degron pathway (N-recognins). This implies a certain accessibility, flexibility, and favorable charge, as well as hydrophobic values, of the enzymatically accessible N terminus, in addition to the initiator amino acid, and also of the residues following the neo–N terminus of the C-terminal protein fragment after cleavage. In short, for being processed by the N-degron pathway cascade, this N terminus should not interfere with binding to the N-degron pathway enzymes. To be processed further, internal Lys residues that can serve as Ub acceptors are needed during polyubiquitination. Together these are critical hallmarks of N-terminal degrons. One decisive step is recognition of N-degrons by E3 Ub ligases, which in turn associate with Ub-conjugating enzymes (E2) carrying Ub that was previously activated by E1 enzymes. Abbreviation: X, any N-terminal amino acid. The surface representations are based on the X-ray structure (PDB ID: 1U70) of murine dihydrofolate reductase (DHFR), which served as a protein model, and were modified and visualized with PyMOL.

the context of the Cys/Arg branch. I also discuss modification of proteins with N-terminal Cys residues and of the residues themselves, where shown to be relevant to the Cys/Arg branch, covering the phenotypes of the corresponding *pco*, *ate*, and *prt6* mutants in plants. Both ATEs and PRT6 are thought to interact with and thus enzymatically modify N termini that are different from those directly related to the Cys/Arg branch (i.e., Cys and its derivatives including arginylated Cys).

PLANT CYSTEINE OXIDASE (PCO): plant pioneer enzyme family of dioxygenases that specifically oxidize N-terminal Cys residues to Cys sulfinic acid

ARGINYL-TRANSFER RNA (tRNA):PROTEIN ARGINYLTRANSFERASEs (ATEs): function appears to be conserved in plants, yeast, and animals; multigene family in plants; in *Arabidopsis*, ATE1 is active in vitro and ATE2 is not

Rumors About Cys Oxidation and Arginylation

The primary research literature (45, 84, 96), many early (15–17, 22) and recent reviews (8, 9, 33, 43, 57, 105, 118), and textbooks (3, 113) have been hypothesizing about PTMs by N-degron pathway enzymes for many years. In most cases, this was based on one or more of the following effects: (*a*) degradation that is dependent on the identity of the N-terminal amino acid, (*b*) degradation that is dependent on stabilization of potentially instable proteins in N-degron pathway mutant plants or cell lines, (*c*) replacement of the possibly destabilizing N-terminal residue by a stabilizing one, and (*d*) the simple theoretical possibility of having to deal with an instable protein. Often, these effects were presumed to follow one of several possible steps of biochemical modification with the mindboggling effect of having to deal with a disappearing protein in vivo or in vitro, and are matters of intense discussions in the field (27, 28).

Breaking Down the Rule into Distinct Branches

Description of the Cys/Arg branch of the N-degron pathway is intrinsically linked with the requirement for Cys oxidation. This specific process, including N-degron pathway–mediated

protein degradation, has been directly associated with cardiovascular development in mammals (65, 80, 82) since work by the Varshavsky laboratory in the late 1990s (24) and following two breakthrough papers that were published in late 2011 by the Holdsworth (45) and van Dongen (84) laboratories on plant environmental stress response during hypoxia.

NOVEL ROLES OF THE N-DEGRON PATHWAY

It has not yet been ten years since novel physiological roles of the N-degron pathway were discovered in plants, and in this period, several milestones concerning the biological function, biochemical mechanisms, and physiological requirements of this pathway were accomplished. The functions—still restricted to a handful of substrates—are mainly associated with stress physiology and targeted degradation of regulatory proteins under abiotic and biotic stress conditions. Targets of the Cys/Arg branch of the N-degron pathway for which protein N-terminal modifications have been described on the molecular level are transcription factors belonging to group VII of the B-2 subfamily of ETHYLENE RESPONSE FACTORs (ERFVIIs). Their conditional stability and accumulation, which are caused by low oxygen levels and related to their N-terminal initiator amino acid [Cys after N-terminal Met excision (NME)], and their degradability, which is dependent on the following highly conserved N-terminal protein sequence motif and the presence of a functional, nonmutated, repertoire of N-degron pathway enzyme–coding genes, were shown to be involved in physiological responses to hypoxic environmental conditions. Hypoxia—in the context of stress physiology rather than as a matter of organ and tissue development (62) behind hermetically separated, oxygen-tight cellular structures—occurs as a consequence of plant submergence in water and waterlogging (41, 45, 84, 96, 107, 130); water stress, such as during drought; and osmotic stresses after exposure to high salinity (88, 124) (**Figure 2**; **Table 1**). In *Arabidopsis*, the ERFVII family of transcription factors consists of five members: RELATED TO APETALA 2.2 (RAP2.2), RAP2.12, RAP2.3 [also known as ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN (EBP)], HYPOXIA-RESPONSIVE ETHYLENE RESPONSE FACTOR 1 (HRE1), and HRE2. These ERFVIIs and their orthologs in other plant species share the highly conserved N-terminal degron (N-degron) motif MCGGAI/V (in single-letter amino acid code), which plays a central role in N-degron pathway–mediated protein turnover.

Only recently have a greater number of functions and applications of the N-degron pathway in plants been described; however, these data do not explicitly address the highly specific Cys/Arg branch of the N-degron pathway nor do they suggest that this branch is involved (28). Examples are of plant–pathogen perception and response (25, 58, 125), cell differentiation and organ development (32), and conditional genetics by targeted protein degradation harnessing the N-degron pathway system in vivo (26, 35–38).

FUNCTIONS OF THE N-DEGRON PATHWAY IN PLANTS

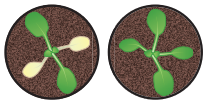
According to the phenotypes of N-degron pathway mutant plants, the pathway regulates multiple complex developmental processes throughout the life cycle (43, 44, 57) (**Figures 2** and **3**). Its enzymatic components are associated with the following functions: seed ripening, lipid breakdown, hormonal signaling, germination (63, 107, 139), leaf and shoot morphogenesis, flower induction, apical dominance (56), delay in senescence (137), and cell division (32, 56). A prominent insight during the past decade was that the N-degron pathway functions as sensor for reactive oxygen species (ROS) and reactive nitrogen species and regulates stress response after hypoxia, e.g., after flooding and plant submergence and during waterlogging (45, 84, 88) (see the sidebar titled Hypoxia Sensing in Animals). The pathway was also shown to be involved in growth-related processes in the moss *Physcomitrella patens*, where mutation of the single *ATE* (dlate) caused delayed

PROTEOLYSIS 6 (PRT6): candidate E3 Ub ligase that restores the ability to degrade the Arg-initiated GUS reporter protein in, and thus functionally complements, the *S. cerevisiae* ubr1Δ mutant

Group VII of the B-2 subfamily of ETHYLENE RESPONSE FACTORs (ERFVIIs): five-member B-2 subfamily of transcription factors consisting of RAP2.2, RAP2.3, RAP2.12, HRE1, and HRE2

N-terminal Met excision (NME): initiator Met residue is cotranslationally cleaved off at the N terminus if the second (penultimate) residue is small and uncharged but not if it is bona fide destabilizing according to the N-end rule (N-degron pathway)

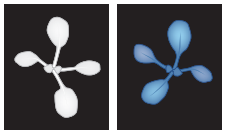
a Delayed senescence



Wild type *ate*

Yoshida et al. 2002

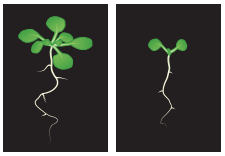
b Stabilization of R-GUS



Wild type *prt6*

Garzón et al. 2007

c Reduced germination



Wild type *prt6*

Holman et al. 2009

d Disturbed development

Wild type *ate1 ate2*

Graciet et al. 2009

e Higher hypoxia tolerance

Wild type *ate1 ate2* *prt6*

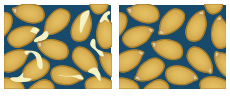
Gibbs et al. 2011

f Lower submergence tolerance

Wild type *prt6* *ate1 ate2*

Licausi et al. 2011

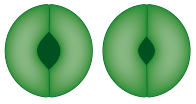
g Reduced germination



Wild type *prt6*

Gibbs et al. 2014b

h Reduced stomata aperture



Wild type *prt6*

Gibbs et al. 2014b

i Reduced plant growth

Wild type *pco1 pco2*

Weits et al. 2014

j Higher submergence tolerance

Wild type *35S::PCO1* *prt6*

Weits et al. 2014

k Higher waterlogging tolerance

Wild type *prt6*

Mendiondo et al. 2016

l Greater sensitivity to ABA during germination

Wild type *prt6*

Riber et al. 2015

m Increased survival under darkness

Wild type *prt6*

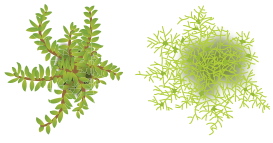
Riber et al. 2015

n Higher susceptibility to root gall disease

Wild type *prt6*

Gravot et al. 2016

o Delayed developmental transition from protonema to gametophore



Wild type *d|ate*

Schuessele et al. 2016

p Dwarfed growth and abnormal leaves

Wild type *d|ate*

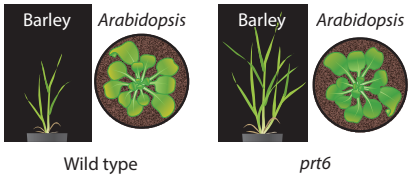
Schuessele et al. 2016

q Delayed chlorophyll degradation

Wild type *d|ate*

Schuessele et al. 2016

r Increased tolerance to salinity



Wild type *prt6*

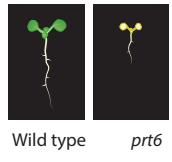
Vicente et al. 2017

s Increased tolerance to drought

Wild type *prt6*

Vicente et al. 2017

t Increased sugar sensitivity



Wild type *prt6*

Zhang et al. 2018

(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Phenotypes of mutants impaired in the Cys/Arg branch of the N-degron pathway. Loss-of-function mutants in the Cys/Arg branch of the N-degron pathway have been compared under different developmental and environmental conditions. Phenotypes are from *Arabidopsis* mutants unless otherwise noted. (*a*) A mutant for ARGINYLTRANSFERASE 1 (ATE1) in the Wassilewskija-0 (Ws-0) ecotype of *Arabidopsis*, which is lacking transcript of ATE2 (due to a point mutation that generates an early stop codon), is delayed in senescence. This led to the naming of the *ate1* mutant in Ws-0 background as *delayed leaf senescence 1* (*dls1*) (137). (*b*) The artificial Arg-initiated ubiquitin fusion technique (UFT) (106) reporter R-GUS was stabilized in *proteolysis 6* (*prt6*) mutants, as identified by sequence homology (40). (*c*) Germination ability was reduced in *prt6* mutants (63). (*d*) Development was widely disturbed on multiple levels in *ate1 ate2* and *prt6* mutants (56). (*e*) Tolerance to hypoxia (flushing with nitrogen on in vitro–grown seedlings) was increased (45). (*f*) Tolerance to hypoxia (submergence in water of soil-grown plantlets) was decreased (84). The conflicting results of panels *e* and *f* arise from different experimental conditions and treatments, as discussed elsewhere (8, 107, 110). (*g*) Germination was reduced upon NO treatment in *prt6* mutants (46). (*h*) Stomata aperture (opening) was reduced in *prt6* mutants (46). (*i*) Plant growth was reduced in *pco1 pco2* double mutants (130). (*j*) Submergence tolerance was reduced upon overexpression of PLANT CYSTEINE OXIDASE 1 (PCO1) and PCO2 and increased in *prt6* mutants (130). In panel *j*, higher submergence tolerance given by the *prt6* or *ate1 ate2* loss-of-function mutations represents a mimicry of PCO misexpression (35S::PCO1). PCOs should be considered epistatic of PRT6 and ATE1 and possibly also of ATE2 (no function shown to date). (*k*) Tolerance to waterlogging was increased in barley with reduced levels of PRT6 [RNA interference (RNAi)] and in TILLING lines (88). (*l*) During germination, *prt6* mutants showed greater sensitivity to abscisic acid (ABA) compared with the wild type (107). (*m*) *prt6* mutants have increased survival under darkness, characterized by isolation of the novel *prt6* allele *greening after extended darkness1* (*ged1*) (107). (*n*) *ate1 ate2* and *prt6* mutants show an increased susceptibility to root gall disease induced by the clubroot agent and protist *Plasmodiophora brassicae* (58). (*o*) The *ATE* mutant d|ate of the moss *Physcomitrella patens* is developmentally arrested in the protonema stage and (*p*) develops dwarfed and malformed shoots after several months, and (*q*) d|ate mutant plants stay green longer after prolonged cultivation in the dark in contrast to the wild type (111). (*r*) Tolerance to salinity was increased in both *Arabidopsis prt6* and barley with reduced levels of PRT6 (RNAi) (124). (*s*) Tolerance to drought was increased in barley with reduced levels of PRT6 (RNAi) (124). (*t*) *prt6* mutants are reduced in oil body breakdown following germination and are hypersensitive for sugar during seedling establishment (139).

gametophore development and growth. The moss d|ate mutants formed caulonema and showed normal gravitropism but were apparently strongly delayed in chlorophyll breakdown (111).

PRT1 N-Degron and PRT6 N-Degron Pathways: Named for N-Recognin

PROTEOLYSIS 1 (PRT1) was found to be required for degradation of Phe-initiated reporter constructs (6, 103, 115). The type II N-recognin E3 Ub ligase exhibits a highly specific ubiquitination activity toward test proteins initiated with bulky hydrophobic N-terminal amino acids in vitro (75, 90, 92) and in vivo (32). So far in plants, only two E3 ligases, PRT1 and the type I N-recognin PRT6, are clearly associated with the N-degron pathway (**Figures 1** and **2**). Polyubiquitination mediated by PRT6 still has to be shown, but PRT6 is treated as an E3 Ub ligase due to the results of heterologous complementation in yeast (40) and plants (84, 88) (see the sidebar titled PRT1 N-Degron and PRT6 N-Degron Pathways).

As a type II N-recognin, PRT6 is per definition one of the decisive enzymes of the Arg/N-degron pathway. Since, in plants, the function of the *Saccharomyces cerevisiae* dual specificity N-recognin UBR1 is diversified into the two (or more yet undiscovered) E3 Ub ligases PRT1 and PRT6, the "N-end rule community" suggests using the terms PRT1 N-degron pathway and PRT6 N-degron pathway to distinguish between targets initiated with type II versus type I destabilizing residues at the N-end (see 89, 121).

Arginyltransferases: Genetic Phenotypes

PRT1 was characterized as the first bona fide plant N-degron pathway E3 Ub ligase (103, 115) during the same period that ATE function was first described in plants. The *delayed leaf senescence 1* (*dls1*) mutant carried a mutation in the *ATE1* gene in the background of the *Arabidopsis* accession Wassilewskija-0 (Ws-0) (137). The mutant plants showed age-dependent senescence delayed in the leaves. Additionally, N-degron pathway activity of the Arg branch was tested in isolated plant

PROTEOLYSIS 1 (PRT1): plant pioneer E3 Ub ligase for native and artificial substrates initiated by the three primary destabilizing bulky hydrophobic type II residues, Phe, Tyr, and Trp

PRT1 N-degron pathway: formerly considered part of the Arg/N-degron pathway but targets substrates initiated with the three hydrophobic residues, Phe, Tyr, and Trp, unrelated to Arg

PRT6 N-degron pathway: formerly listed as a major part of the Arg/N-degron pathway that would target the three primary destabilizing basic type I residues, Arg, Lys, and His

Table 1 Genes of the Cys/Arg branch of the N-degron pathway, their known functions, and phenotypes of mutants

Gene	Phenotype(s) of mutants	Reference(s)
Development		
ATEs	Delayed leaf senescence	137
PRT6	Slower germination and lipid breakdown, hypersensitive against ABA during germination	63[a]
ATEs, *PRT6*	Abnormal leaf and shoot development	56[a]
ATEs	Delayed gametophore development, growth and prolonged greening in the moss *Physcomitrella patens*	111
PCOs	*pco1 pco2* double mutant slightly affected in general plant growth under normoxic conditions	130
PRT6	Increased sugar sensitivity and reduced oil body breakdown during germination	139
Abiotic stress		
PRT6	More resistant to hypoxia[b]	45[a]
	Less resistant to submergence[b]	84[a]
	Reduced germination after NO treatment; reduced stomatal closure	46
	Increased survival under darkness, lead to the identification of novel *prt6* allele *greening after extended darkness1* (*ged1*)	107
	Sensitive to ABA during germination	107
	More tolerant to waterlogging in barley	88
	More resistant to salinity and drought in *Arabidopsis* and barley	124
Biotic stress		
ATEs	Misregulation of defense- and JA-responsive genes	25
ATEs, *PRT6*	Less resistant to pathogens[c]	25
	Enhanced susceptibility to root gall disease	58
	More resistant to pathogens[c]	125
Molecular[d]		
PRT6	Accumulation of Arg-initiated GUS (R-GUS) artificial reporter protein[d]	40
ATEs	Accumulation of Met-, Gln-, Asn-, Cys-, and Asp-initiated Luc[d]	55

[a]Published back-to-back: Holman et al. 2009 (63) and Graciet et al. 2009 (56), and Gibbs et al. 2011 (45) and Licausi et al. 2011 (84).
[b]Results discussed, e.g., in References 8, 107, and 110.
[c]Results discussed, e.g., in Reference 125.
[d]For clarity, only one molecular phenotype is shown for the stability reporter R-GUS that is relevant for the Arg/Cys branch of the N-degron pathway. Other N-degron pathway substrate-related stability reporters were used in the context of ERVII and VRN2 characterization, for example. All these data are cited in the text.
Abbreviations: ABA, abscisic acid; ATE, ARGINYLTRANSFERASE; JA, jasmonic acid; PCO, PLANT CYSTEINE OXIDASE; NO, nitric oxide; PRT6, PROTEOLYSIS 6.

Neo–N terminus: N-terminal amino acid residues and their posttranslational modifications, which are different from those of the native or nascent protein N terminus

cells (protoplasts) of the *dls1* mutant. Asp-initiated and Glu-initiated firefly luciferase (Luc) reporter proteins were instable in the *dls1* mutant, whereas Met at the resulting neo–N terminus of a Ub-fusion-technique construct (92, 106, 133) led to a longer half-life. Of note, *ATE1* and *ATE2* in the *Arabidopsis* genome encode for two candidate ATEs. The Ws-0 accession, however, carries a single-nucleotide insertion in the *ATE2* gene that leads to a premature stop codon and, consequently, to a presumably truncated and nonfunctional ATE2 protein. Thus, Ws-0 contains only one functional *ATE* gene compared with the often-used Columbia-0 (Col-0) reference accession (107). Only recently, ATE1 but not ATE2 was found to be active in in vitro Arg-transfer reactions when prepared as recombinant protein from bacteria (132, 140).

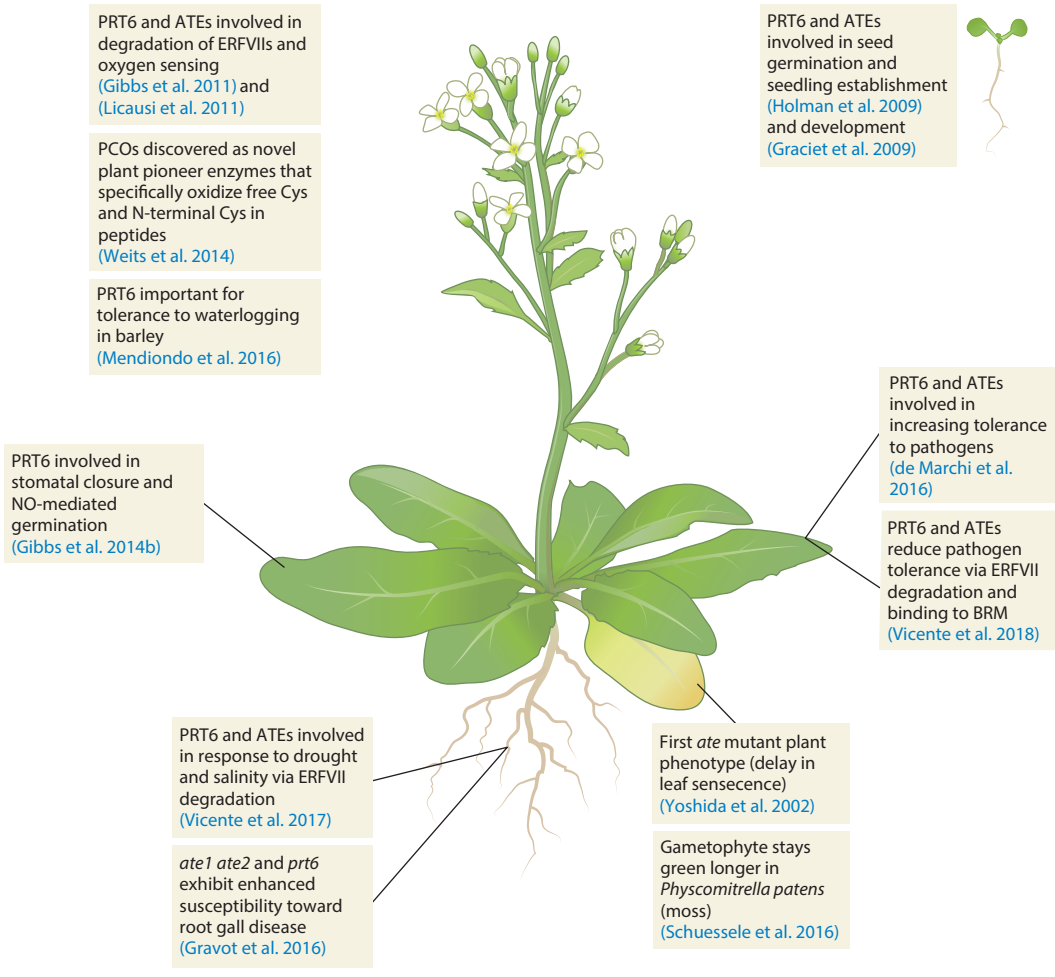


Figure 3

The Cys/Arg branch of the N-degron pathway in stress response. The Cys/Arg branch of the PRT6 N-degron pathway was shown to function primarily in oxygen sensing and hypoxia response. Recently, the branch was found to be involved in response to plant pathogens, including innate immunity, and also to have impact in general plant development and growth performance. Findings pertain to the following references: Yoshida et al. 2002 (137), Holman et al. 2009 (63), Graciet et al. 2009 (56), Gibbs et al. 2011 (45), Licausi et al. 2011 (84), Weits et al. 2014 (130), Gibbs et al. 2014b (46), de Marchi et al. 2016 (25), Mendiondo et al. 2016 (88), Vicente et al. 2017 (124), Vicente et al. 2018 (125). (Note: all studies involved *Arabidopsis* unless otherwise noted in the figure.) Abbreviations: ATE, ARGINYLTRANSFERASE; BRM, BRAHMA; ERFVII, group VII of the B-2 subfamily of ETHYLENE RESPONSE FACTORs; PCO, PLANT CYSTEINE OXIDASE; PRT6, PROTEOLYSIS 6.

Arginyltransferases: Molecular Phenotypes and Biochemistry

Extracts of rice and wheat embryos were shown to contain an activity of aminoacyl transfer that was able to transfer amino acids from tRNA to a protein acceptor (87). Arg was metabolized, but the nature of the enzyme and the acceptor position in the proteinaceous cosubstrate remained unclear. This led to the speculation that the N terminus could serve as an acceptor, as was previously described in mammalian cells (72). Asp and Glu were determined as the acceptor residues (112). However, the function and biological role of this arginyltransfer activity continued to remain unclear for decades (73).

Ub fusion technique: exposes N-terminal amino acids of choice by fusing an N-terminal Ub moiety N-terminally to the desired residue

HYPOXIA SENSING IN ANIMALS

In animals, a homeostatic balance of O_2 levels and adaptation to hypoxia is mediated by the hypoxia-inducible transcription factors (HIFs). In normoxic conditions, HIFs are hydroxylated at specific proline residues, targeting them for binding to an E3 Ub ligase, which results in HIF ubiquitination and proteasomal degradation. HIFs are not substrates of the N-degron pathway and do not undergo Cys oxidation, but their ubiquitination is sensitive to hypoxia. HIF prolyl hydroxylation is catalyzed by O_2-dependent enzymes, i.e., the HIF prolyl hydroxylases that are highly sensitive to O_2 availability. The HIF hydroxylases are Fe(II)/2OG-dependent oxygenases. The PLANT CYSTEINE OXIDASEs are the first Cys dioxygenases to be identified in plants.

Arginylation: posttranslational modification mediated by ATEs that mostly occurs via a peptide bond at the N-terminal alpha-amine group of proteins

The first hint about the molecular function of ATEs in *Arabidopsis* came from the use of highly purified plant extracts in an in vitro arginylation assay. Treated extracts of the wild type and *ate1 ate2* double mutant, both in the background of the Col-0 accession (harboring two transcribed gene copies of *ATEs*), were incubated with the artificial protein substrate bovine α-lactalbumin, an experimental Arg acceptor protein. Incorporation of radiolabeled Arg was seen for the reaction containing the wild-type extract but not for the *ate1 ate2* double mutant (56). It remained unclear if the transferase activity might have been lost already in the *ate1* single mutant as suggested by later sequencing results of the ATE2-lacking Ws-0 accession (107). Soon after this, *Arabidopsis* ATEs were expressed in an *S. cerevisiae ate1Δ* mutant, and it was shown that this was sufficient to destabilize the artificial reporter protein β-galactosidase (β-Gal) if initiated with Asp or Glu. Therefore, the presence of ATEs was seen to be required for destabilization of Asp-initiated or Glu-initiated reporters, but the mechanism of this effect remains unclear (55).

WHAT MAKES A PROTEIN A TARGET?

Potential degradation signals of the N-degron pathway are mostly buried in the complete, unprocessed sequence of the bona fide targets or are otherwise cryptic. Activation of an N-degron pathway substrate may, according to the current understanding, be generated by three means: (*a*) endoproteolytic cleavage of proproteins and preproproteins, possibly in proteolytic cascades

PRT1 N-DEGRON AND PRT6 N-DEGRON PATHWAYS

In animals, the N-degron pathway consists of three branches, the Ac/N-degron pathway (acetylation), the Arg/N-degron pathway (arginylation), and the Pro/N-degron pathway (mediated via N-terminal Pro). In plants, it remains to be determined whether there are Ac and Pro pathways. Then, the Arg/N-degron pathway is diversified and functionally operated by at least two N-recognins, PRT1 and PRT6. PRT1 is assumed to bind only to the three hydrophobic bulky type II residues, Phe, Tyr, and Trp, but has nothing to do with recognition of Arg residues (**Figure 1**); PRT6 is assumed to bind to all type I residues, Lys and His, as well as Arg, which are all positively charged. Therefore, PRT6 may also operate on secondary and tertiary destabilizing residues via preceding deamidation or deoxygenation followed by arginylation (**Figure 1**). PRT1 was associated with the Arg/N-degron pathway only for historic reasons. For clarity, the plant Arg/N-degron pathway has now been separated into the PRT1 N-degron and PRT6 N-degron pathways. The Cys/Arg branch of the N-degron pathway specifically addresses the cascade starting from exposure of N-terminal Cys residues followed by oxidation (oxygenation via PCOs), arginylation via ATEs (at least ATE1), and polyubiquitination and degradation by the proteasome (both of which have yet to be demonstrated).

(97), revealing a previously latent degradation signal from within the original complete protein sequence at the freshly formed C-terminal cleavage fragment (**Figure 4**), (*b*) NME during which nascent proteins cotranslationally lose their initiator Met residue and therefore expose the originally second amino acid as the neo–N terminus (**Figure 4**), and (*c*) N-terminal acetylation, which may activate otherwise stabilizing N-terminal amino acids as degradation signals. This means that N-degron pathway substrates are considered to be generated posttranslationally by a series of related biochemical events happening to otherwise stable proteins (29, 97).

Met aminopeptidases (MAPs): highly conserved members of one of two classes of MAPs (MAP1 and MAP2) that cleave initiator Met residues during NME in eukaryotes

Nitric oxide (NO): a reactive oxygen species or reactive nitrogen species that generates proteotoxic stress, renders reporter proteins with N-terminal Cys instable, and is therefore suggested to cause oxidation of N-terminal Cys

A HIERARCHICAL BIOCHEMICAL REACTION CASCADE

Certain PTMs involving N-terminal processing can generate N-degron pathway substrates from precursors, as described previously. These precursors are mostly proproteins and preproproteins that get modified after translation. The very first step, the priming of targets, may occur either via NME by Met aminopeptidases (MAPs) (18, 48, 52), which is cleavage by endopeptidases leading to the production of destabilizing residues at the freshly formed N terminus of the C-terminal fragment (**Figure 4**), or via acetylation (42, 67). The neo–N termini that result after NME (cleavage) may represent any of the 20 proteinogenic amino acid residues. Only some of them (**Figures 1** and **5**) will have the biochemical properties to be either directly recognized by N-recognins or further modified by downstream enzymes. In the Arg/N-degron pathway, the hierarchy is best explained by the PTM of protein N termini that do not carry any of the type I primary destabilizing residues, Arg, Lys, and His, or type II primary destabilizing residues, Phe, Tyr, Trp, Leu, and Ile. Instead, these N termini might be initiated with other so-called secondary destabilizing residues (Nd^s), such as Asp and Glu, or tertiary destabilizing residues (Nd^t), such as Asn, Gln, and Cys. In later sections, Cys is discussed in depth given its pivotal role in the Cys/Arg branch of the N-degron pathway.

TO BE OR NOT TO BE DESTABILIZING?

Further metabolization of tertiary or secondary destabilizing residues at the neo–N termini of prospective N-degron pathway targets can be achieved by oxidation, deamidation, and arginylation. These Nd^ts and Nd^ss can be converted to arginylation-permissive pro-N-degrons. For example, in mammals and mammalian cell culture, N-terminal Cys residues, which are tertiary destabilizing, can be subject to nonenzymatic oxidation (24, 65, 80, 82) (**Figure 5**). In plants, these residues were shown to be subject to both nonenzymatic oxidation by nitric oxide (NO) (46) and enzymatic oxidation by PCOs (130–132) (**Figure 6**). Deamidation of N-terminal Gln and Asn, which, like Cys, are tertiary destabilizing residues, is a way to generate the two secondary destabilizing residues Asp and Glu. This was shown in *S. cerevisiae* by action of the dual-specific N-terminal amidase NTA1p (11) and in mammals by the Asn-specific N-terminal amidase (NTAN1) (59, 79) and the Gln-specific N-terminal amidase (NTAQ1) (128) (**Figure 1**). Plants do not contain any homologs with significant sequence similarities to *S. cerevisiae* NTA1p, but putative orthologs of Ntan1 and Ntaq1 from mouse were found. *S. cerevisiae nta1* mutants (11) that were heterologously complemented with the cDNAs of the putative NTAN1 and NTAQ1 from *Arabidopsis* were able to degrade artificial Asn-initiated or Gln-initiated reporter proteins (55).

The next potential step in the N-degron pathway cascade of PTMs is arginylation, which can enable recognition of targets by N-recognins via posttranslationally added Arg residues that are themselves primary destabilizing residues, to the α-amino group of acidic Asp or Glu residues at the N terminus (12, 54, 80).

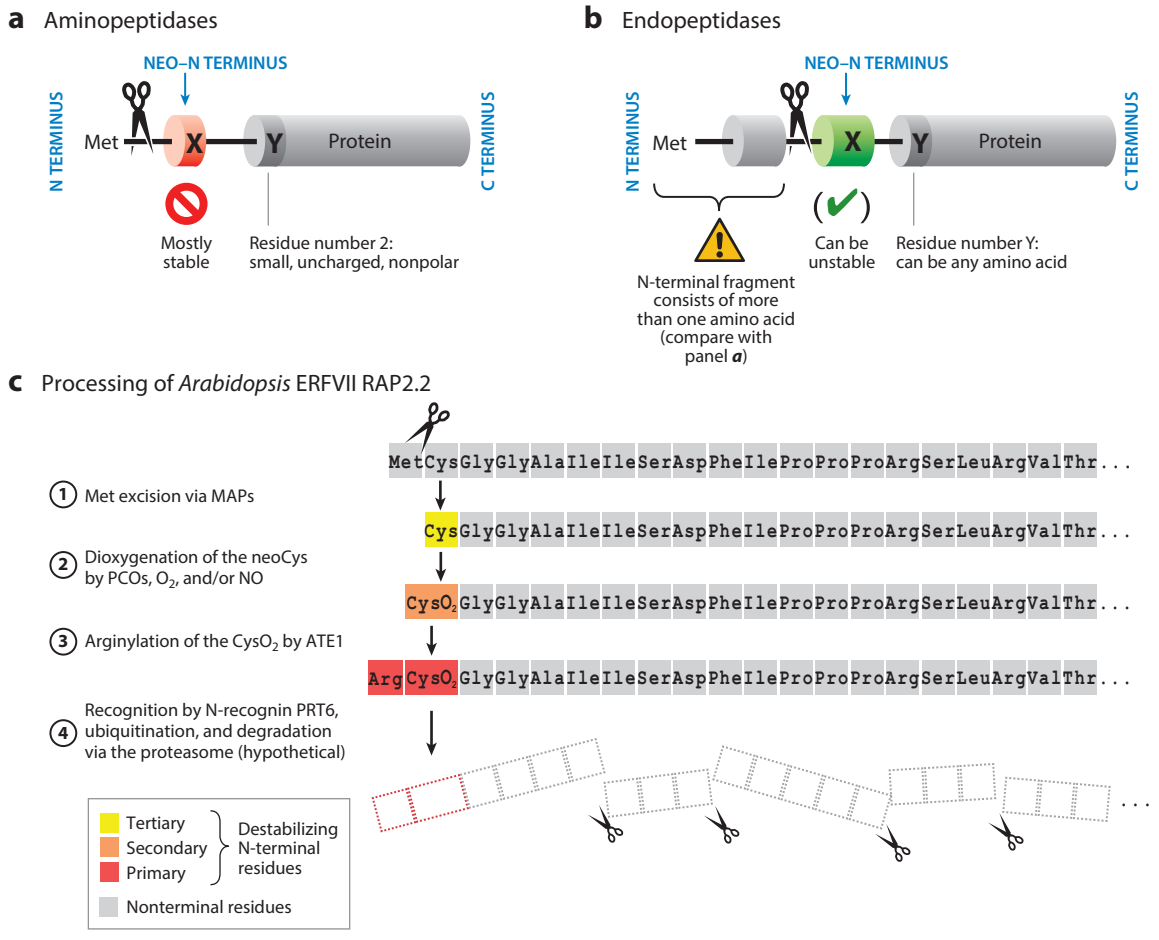


Figure 4

The making of N-degron pathway targets. Bona fide N-degron pathway substrates, i.e., proteins containing potential N-terminal degrons (N-degrons), can be generated after proteolytic cleavage from proproteins and preproproteins that serve as precursor sequences. N-degrons are defined as neo-N-terminal residues that get recognized by the E3 ubiquitin (Ub) ligases of the N-degron pathway (N-recognins). This implies the requirements of a certain accessibility and/or flexibility, a favorable charge, and hydrophobic values that would not interfere with binding to the N-degron pathway enzymes. To be processed further, internal Lys residues are needed for polyubiquitination. N-degrons can then be recognized by E3 Ub ligases, which in turn associate with Ub-conjugating (E2) enzymes carrying Ub that was previously activated by E1 enzymes. (*a*) Substrates can be generated by N-terminal Met excision (NME) via action of Met aminopeptidases (MAPs) that cotranslationally cleave just the initiator Met residue of the nascent protein. This potential entry to the N-degron pathway seems to be reserved for Met-Cys-initiated proteins in plants because they are the only ones cleaved to result in the neo–N terminus carrying the (tertiary) destabilizing residue, Cys. In yeast, NME also occurs on nascent proteins bearing Met-Asn or Met-Gln at their N termini (94). (*b*) The second pathway to generate substrates is endoproteolytic cleavage by proteases, in which longer fragments of the previous N terminus of the (pre-)proprotein are cut off. This includes transit and signal peptides, inhibitory domains, and other subdomains. (*c*) Processing of RELATED TO APETALA 2.2 (RAP2.2), one of the five members of the group VII of the B-2 subfamily of ETHYLENE RESPONSE FACTORs (ERFVIIs) transcription factors, according to the contemporary understanding, is composed of individual steps of a biochemical cascade: relevant for Cys-Arg/N-degron pathway are (①) methionine excision by MAPs; (②) cysteine dioxygenation by PLANT CYSTEINE OXIDASEs (PCOs), molecular oxygen (O_2), and/or nitric oxide (NO); (③) arginylation of dioxygenated Cys by ATE1; and (④) recognition by N-recognin PROTEOLYSIS 6 (PRT6) and possibly other recognition enzymes, followed by ubiquitination and degradation via the proteasome. Evidence for polyubiquitination and binding by PRT6 is still lacking; however, the molecular priming of Met-excised RAP2 (132) and VRN2 (47) N termini via PCOs through dioxygenation of the neoCys, and in the case of RAP2, followed by arginylation of the Cys sulfinic acid by ARGINYLTRANSFERASE 1 (ATE1) (132) was recently shown.

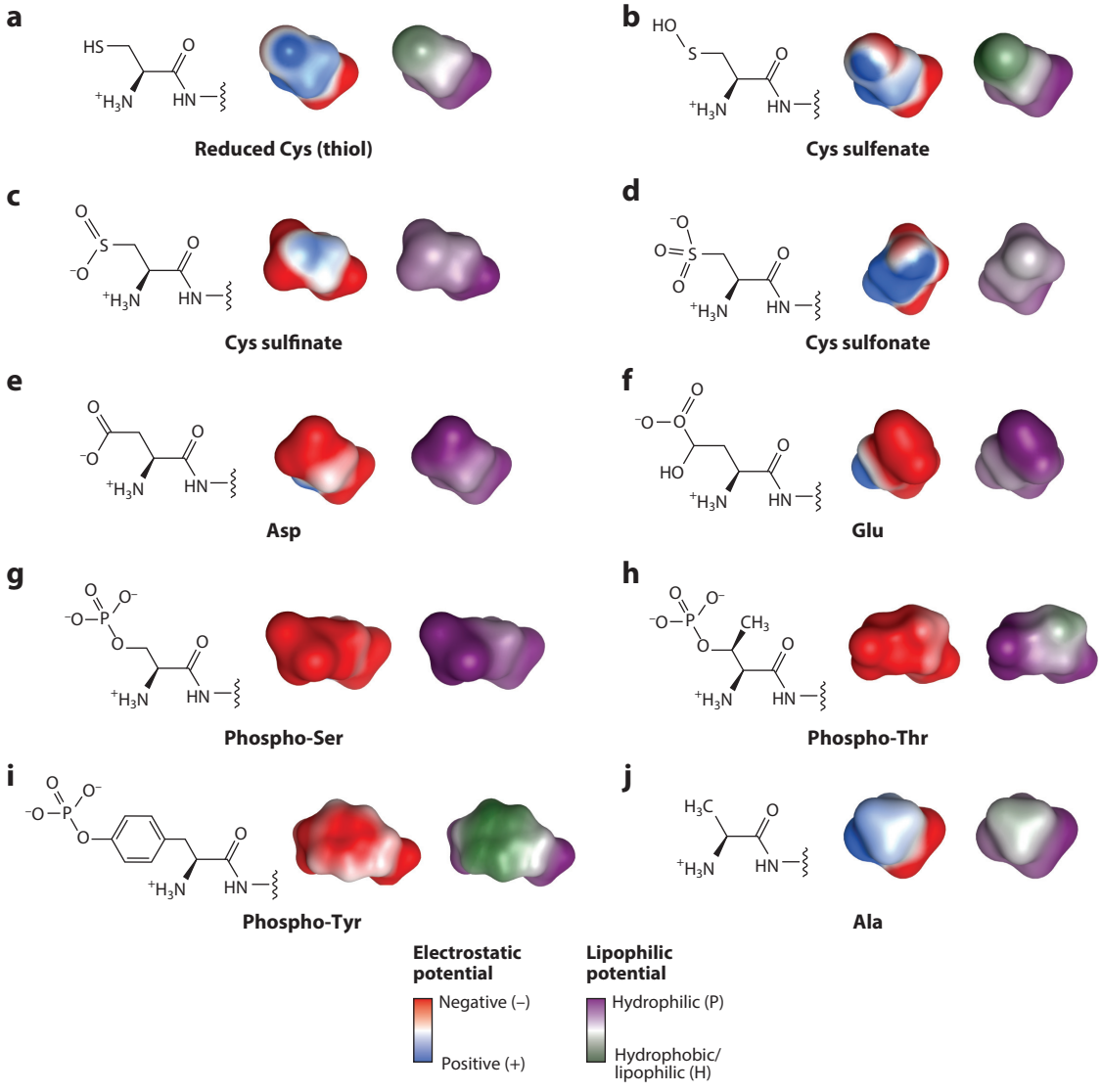


Figure 5

Similarity of oxidized Cys and negatively charged native or posttranslationally modified proteinogenic amino acids. The amino acids are drawn in their N-terminal, $^{+}H_3N$-initiated, and peptide-bonded (−CONH−) form with the N terminus facing the left. Wave line marks the junction to residue number two. Heterogeneous surfaces are shown next to the skeleton representation (*left*) in the same orientation and colored according to the electrostatic (*center*) or the lipophilic (*right*) surface potentials. A comparison of (*a*) Cys (thiolate) with (*b*) Cys sulfenate, (*c*) Cys sulfinate, (*d*) Cys sulfonate, (*e*) Asp, (*f*) Glu, (*g*) phospho-Ser, (*h*) phospho-Thr, (*i*) phospho-Tyr, and (*j*) Ala, which is commonly used to replace Cys residues in Met-Cys-initiated proteins to test potential stabilization. Asp and Glu are commonly used for phosphomimetic substitutions; note the similarity to phospho-Ser and phospho-Thr and especially to the various oxidized Cys derivatives. For the electrostatic potential, by convention, red corresponds to negative (–) potential and blue corresponds to positive (+) potential. For the hydrophobic/lipophilic potential, green represents hydrophobic (H) and lilac represents hydrophilic (P). The surfaces were prepared using Molecular Operating Environment (MOE; Chemical Computing Group, version 2018.01) and the Spartan'18 Parallel Suite (Wavefunction, Inc.).

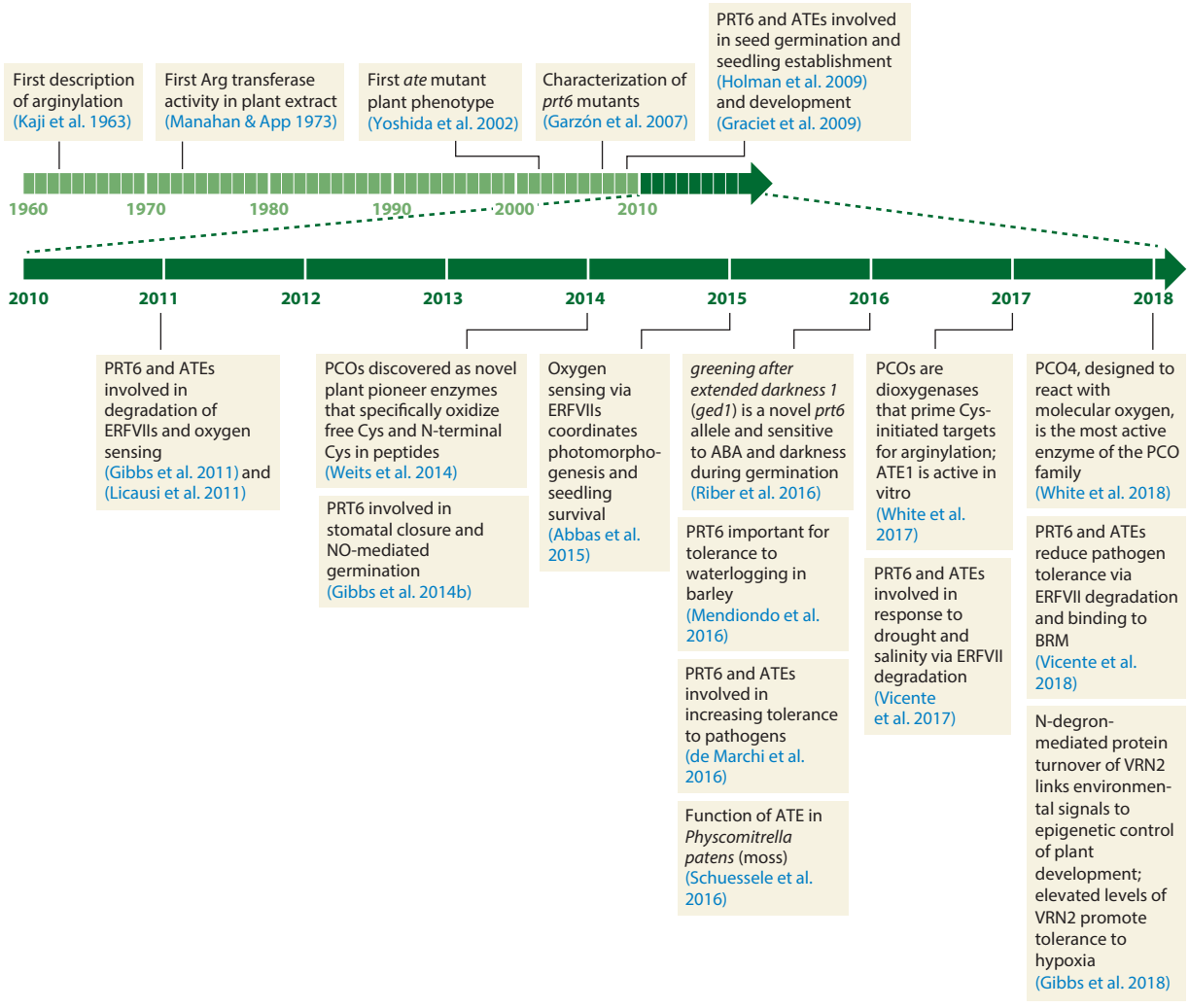


Figure 6

Timeline of discovery for the Cys/Arg branch of the N-degron pathway. The pace of research related to the Cys/Arg branch of the N-degron pathway has greatly accelerated over the last decade since novel physiological roles were first discovered. During this period, biological functions, biochemical mechanisms, and physiological requirements of the pathway have been elucidated. Abbreviations: ATE, ARGINYLTRANSFERASE; BRM, BRAHMA; ERFVII, group VII of the B-2 subfamily of ETHYLENE RESPONSE FACTOR; PCO, PLANT CYSTEINE OXIDASE; PRT6, PROTEOLYSIS 6.

Again, I wish to highlight that the definition of an amino acid as a destabilizing residue does not directly imply that every protein initiated with that particular residue will be instable and have a short half-life. This has been discussed only marginally in the past (93). Such a candidate might simply represent a compartmentalized protein or a protein whose other determinants for being instable are lacking. Some examples have been described where bone fide N-degron pathway substrate candidates could not be verified as such, namely, some ERFVIIs (45), RIN4 (140), and AtERF#111 (J. Bäumler, W. Riber, M. Klecker, N. Dissmeyer, A. Weig & A. Mustroph, unpublished manuscript).

THE MC-OME

The *Arabidopsis* genome comprises only 246 Met-Cys-starting proteins, a significant underrepresentation compared with the other 19 proteinogenic amino acids. Collectively, these 246 Met-Cys-starting proteins make up the MC-ome, which gets cotranslationally processed by MAPs and loses the N-terminal initiator Met1 residue. Cys is a tertiary destabilizing residue according to the N-degron pathway and needs further posttranslational processing to initiate degradation of its host protein. Cys oxidation represents this critical PTM and can be followed by N-terminal arginylation. Currently, due to a lack of experimental evidence, it is unclear how many Met-Cys-starting proteins are actual substrates of MAPs followed by enzymatic or nonenzymatic oxidation, arginylation, and/or ubiquitination.

THE SPECIAL CASE OF CYS

By default, all N termini of nascent proteins carry Met1 as a standard initiator amino acid. According to current knowledge of the N-degron pathway, MAPs responsible for NME remove this Met1 in cases where the second residue has a small size and is not destabilizing (**Figure 4**). This is due to steric hindrance at the active sites of MAPs. Among the arginylatable residues, only Cys satisfies this condition (48, 120) (**Figure 5**). Only very recently, a study revealed that in yeast, NME also occurs on nascent proteins bearing Met-Asn or Met-Gln at their N termini (94).

Because Cys is a tertiary destabilizing residue that has a small side chain, its exposure is assumed to occur via NME in those proteins starting with Met1 and Cys2 (13, 39, 48, 49). These Met-Cys-starting proteins are known collectively as the MC-ome (see the sidebar titled The MC-ome). N-terminal Cys, like internal Cys residues, can be oxidized as mentioned previously (**Figure 5**). Oxidation of N-terminal Cys of the potential protein degradation target would therefore render the Cys into an arginylation-permissive pro-N-degron by redox modification (**Figure 7**). Chemically, this oxidation can be complete or incomplete. The initial product is reversible monooxidation of a Cys thiol group to a Cys sulfenic/sulphenic acid group (Cys sulfenate, Cys-SOH, or CysO–) by cellular ROS, such as H_2O_2. Sulfenic acids quickly undergo disulfide bond

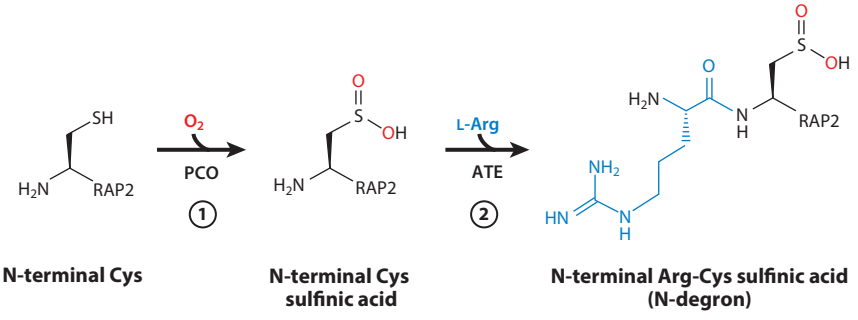


Figure 7

The biochemistry of posttranslational Cys oxidation linked to arginylation. N-terminal Cys residues can be dioxygenated by PCO1 or PCO4 (EC 1.13.11.19) using O_2 as a cosubstrate (①). This leads to formation of a Cys sulfinic acid at the peptide N terminus. The Cys sulfinic acid is, together with cosubstrate L-Arg-tRNA (②), a substrate of *Arabidopsis* ATE1 (EC 2.3.2.8) and results in a novel peptide bond between Arg and Cys sulfinic acid (L-arginyl-CysO_2-protein). Abbreviations: ATE, ARGINYLTRANSFERASE; EC, enzyme class; L-Arg-tRNA, L-arginyl-transfer RNA; PCO, PLANT CYSTEINE OXIDASE; RAP2, RELATED TO APETALA 2.

formation or further oxidation, making detection of sulfenic acids a challenge (108, 135). Irreversible overoxidation or hyperoxidation leads to dioxygenated Cys sulfinic/sulphinic (cysteinic) acid (Cys sulfinate, Cys-SO_2H, or $CysO_2$), and strong oxidizing agents can further oxidize sulfinic acid to fully triple oxidized Cys sulfonic/sulphonic (cysteic) acid (Cys sulfonate, Cys-SO_3H, or $CysO_3$) (85, 105, 129) (**Figure 5**).

Oxidized Cys Is Thought to Accept an N-Terminal Arg Because It Resembles the Negatively Charged Residues Asp and Glu

Cys, in its oxidized state, is now suggested to act as a secondary destabilizing residue. The possible molecular mechanism is a biochemical mimicry of the negative charges and the size of an Asp side chain (80) (**Figure 5**). It is argued that the side chain of Cys sulfinic acid rather than Cys sulfonate more closely resembles the side chain of Asp (126), but one has to keep in mind that in vitro Cys sulfinate is readily oxidized to Cys sulfonate and, depending on the analytics used, Cys sulfonate is produced as a reaction artifact (132). Whether phosphorylated Thr or Ser side chains act as novel phospho-N-degrons has not yet been fully investigated, but the high similarity of Asp and Glu to phospho-Ser and phospho-Thr is widely used in phosphomimetic substitutions (30, 31, 104). Therefore, an interaction with N-degron pathway components could be feasible and open up a totally novel N-degron pathway.

In mammalian and plant systems, it was shown that Cys oxidation can occur both nonenzymatically (46, 65, 82) and enzymatically (130–132). In plants, both NO and O_2 appeared to be required for N-terminal Cys oxidation and protein destabilization of ERFVIIs. ERFVIIs were therefore suggested to act as hubs for the perception of both gases (46).

How Many Oxygens Make an Oxidized Cys?

For roughly two decades, the oxidation state of Cys in the context of the Cys/Arg branch remained elusive. However, it was suggested that the result in vivo is fully oxidized Cys sulfonic acid. Crucial for the plant field was the discovery of plant-specific enzymes, the PCOs, followed by their novel genetic and molecular characterization (130–132). These findings shed light on species-dependent diversifications in the N-degron pathway, where different molecular routes evolved to possibly retain very similar molecular consequences on the level of PTMs and proteostasis, and are intensely discussed elsewhere (27, 28, 121).

OXIDIZED CYS? WHAT'S NEXT?

Cys was once characterized, like Asp and Glu, as "a previously unknown *secondary* [emphasis in original] destabilizing residue" (54, p. 16706) in rabbit reticulocyte lysate, a well-established test system commonly used to determine half-lives of in vitro–translated protein in N-degron pathway studies. This was due to the finding that Cys-initiated test proteins were stable if RNase was contained in the reaction but instable if RNase inhibitor was added to this. This was explained by the requirement of tRNA (Arg-tRNA) to render Cys-initiated proteins, which may have been oxidized previously, instable (54). Later, it was found that arginylation of N-terminal Cys requires preceding oxidation (65). In animals, N-terminal Cys can be oxidized through nonenzymatic reactions that require NO but not O_2 (65).

Setting the Pace: Cys Oxidation in Nonplant Systems

Until very recently, there existed a strong ambiguity in the N-degron pathway literature regarding Cys sulfinic versus Cys sulfonic acid modifications. In 2000, mouse regulator of G-protein

signaling 4 (RGS4) and RGS16, both Met-Cys-starting members of the RGS family of GTPase-activating proteins, were found to be instable when expressed in cell-free rabbit reticulocyte lysates (**Figure 6**). This effect was suggested to be dependent on the possible interplay of N-terminal Cys oxidation followed by arginylation in vitro. That was also the first time that this PTM was documented by chemical Edman degradation (24). Since that time, the role of the penultimate Cys2 residue, which was expected to become the exposed neo–N terminus after NME, and especially the mechanism of its PTM by Ate1, presumably from rabbit reticulocytes in that case, has remained unclear. This uncertainty extends to its chemical state as possibly oxidized—a possibility that was not mentioned in the initial publication. It was stated that the N-terminal initiator Met of RGS4 was replaced with Arg. This is a good simplification that holds true, although N-terminal arginylation appears to remain a rare event. Moreover, until recently, RGS4 and RGS5 (another RGS family member) were the only two cases known where N-terminal Cys is reported to be oxidized and subsequently serves as an arginylation acceptor of ATEs, leading to degradation by the N-degron pathway (82). This recent study described the role and mechanism of Cys modification, writing that "Cys also serves as a pre-N-degron through its oxidation into Cys sulfinic acid ($CysO_2$) or cysteic acid ($CysO_3$)," which was at that point not precisely determined (82, p. 15031). Just before that, fragmentation-based mass spectrometric sequencing of CNBr-produced peptides identified residue 2 of RGS4 as Cys sulfonic acid and suggested an enzymatic oxidation rather than uncatalyzed reaction (80). This is also the only example in the literature where fragmentary mass spectrometry is used to indisputably demonstrate arginylation of a RGS4 peptide with a +48-Da mass consistent with the presence of a fully oxidized Cys (sulfonic acid, 151 Da) at the +2 position (80). A mass of +48 Da corresponds to three O atoms (81), +32 Da corresponds to two O atoms (132), and +156 Da corresponds to arginylation (74, 132).

Incorporation of molecular O_2 has been demonstrated, but nonetheless an enzymatic process is suggested. At the same time, N-terminal arginylation is speculated to function as an O_2 sensor, potentially protecting cells from oxidative stress (80). The latter finding was supported after the stabilities of RGS4 and RGS5 were enhanced in hypoxia (82), but overall this mechanism has not been widely studied.

The gas NO, an important signaling compound in both animals and plants, has been reported to promote Cys sulfonic acid formation and subsequent arginylation at the N terminus of RGS4 and RGS5 in vivo and in vitro via S-nitrosylation. However, both the mechanism and the state of Cys oxidation remain unclear, and molecular evidence has not been provided for nitrosylation or subsequent oxidation (65). This work supports the biological role of NO in vivo by demonstrating stabilizing Met-Cys-initiated substrates in cells lacking NO synthases. In the context of these findings, the in vitro protocols for testing substrates became more elaborate in the animal field, and purified mammalian Ate1 variants from cell cultures could be used to arginylate N-terminal Glu of the reporter protein α-lactalbumin, as verified by Edman degradation. GRP78 (BiP) and protein-disulfide isomerase were also suggested to serve as putative N-degron pathway substrates mediated by N-terminal arginylation (64). However, the three RGS proteins remained the only proteins of the Met-Cys-starting pool; that is, that pertain to the Cys/Arg branch.

Unoxidized N-terminal Cys residues, or more accurately, their deliberate lack of oxidation, make for either very poor arginylation substrates of the mammalian Ate1 or no substrates at all, as tested in peptide arrays. Negligible arginylation was previously seen in cases where the penultimate residue after unoxidized Cys was a positively charged (basic) Lys. It remained unclear if this might also be due to a possible nonenzymatic oxidation to Cys sulfinic acid and/or Cys sulfonic acid by O_2 in the air. Fully oxidized Cys sulfonate was less arginylated compared with Asp at position 1. This was also the first study investigating both first and second position effects on mammalian Ate1 substrate recognition (126). It is important to keep in mind that only Cys sulfonate is

CYS OXIDATION IN ANIMALS

PLANT CYSTEINE OXIDASEs are dioxygenase enzymes. They are similar to mammalian and bacterial Cys dioxygenases (CDOs), to which they show distant sequence homology. The CDO family of enzymes is Fe(II) dependent but does not require external electron donors for O_2 activation. Mouse CDO converts L-Cys to L-Cys-sulfinic acid.

commercially available for peptide synthesis—Cys sulfinic acid is not amenable to preparation by chemical synthesis due to autooxidation. One way to generate Cys sulfinic acid is via enzymatic dioxygenation with PCOs (132) (see sidebar titled Cys Oxidation in Animals).

Shifting Gears: Cys Oxidation in Plants

O_2 sensing via the plant Cys/Arg branch of the N-degron pathway—and therefore via the PRT6 N-degron pathway—impacts the response of organisms to the environment under hypoxic conditions via the oxygen-dependent instability of ERFVII transcription factors (45, 84, 130) (**Figures 2** and **3**; **Table 1**).

In plants, NO regulates important developmental transitions and stress responses and is partially mediated by ERFVIIs. The N-degron pathway was shown to promote the degradation of ERFVIIs upon NO-induced oxidation, happening very likely at the N terminus, and influencing therefore ERFVII-regulated expression of ABSCISIC ACID–INSENSITIVE5 (ABI5), an important downstream transcription factor and key regulator of hormonal crosstalk and abscisic acid (ABA)-mediated stress response (46). N-degron pathway–mediated ERFVII degradation also therefore controls physiological processes, such as seed germination, stomatal closure, and hypocotyl elongation, which are all administered through ABA signaling, highlighting another potential role in stress response. Increased sensitivity against ABA (107) and also against sugar (139) during germination was shown in other studies. PRT6 controls sugar sensitivity of seedling establishment and oil body breakdown following germination (139).

The discovery of PCOs prompted the first close investigation of Cys oxidation. In *Arabidopsis*, there are five members of the PCO family. *PCO1* and *PCO2* are the most frequently expressed *PCO* genes, and their expression has a physiological impact because they are induced by hypoxia. *PCO1* and *PCO2* show close sequence homology, as do *PCO4* and *PCO5*. *PCO3* shows similarities to both groups. PCOs also show some sequence homology to the Fe(II)-dependent Cys dioxygenase (CDO) family of enzymes from mammals. It was shown that PCOs can mediate the oxidation of N-terminal Cys residues to Cys sulfonic acid under consumption of O_2. PCOs could not oxidize an internal Cys residue (130); that O_2 was used as a cosubstrate and actually incorporated into the product was shown later (132). Whether a similar enzyme function involving consumption of molecular O_2 exists in animals is currently unknown (4).

COUPLED CYS OXIDATION–ARGINYLATION IN PLANTS

Behavior of Cys-Initiated Reporters

In plants, Cys-initiated test proteins were shown to be instable, whereas similar reporters in *S. cerevisiae* were shown to be stable. Cys-Luc is short-lived in tobacco leaves and *Arabidopsis* and long-lived in an *Arabidopsis ate* mutant (55). Transplastomic tobacco plants expressing a green fluorescent protein (GFP) in the plastid that was initiated with Met-Cys appeared to be instable

compared with other amino acids at the penultimate N-terminal position after Met (5). Plastid-encoded proteins, like prokaryotic and eukaryotic proteins, were suggested to be posttranslationally processed by N-terminal deformylation and NME (50–52), and removal of Met1 was suggested to influence their stability (52). However, the existence of an N-degron pathway in plastids is still under debate but might resemble an organelle-specific rule that is similar to the one in prokaryotes (5, 14, 29, 109, 138). N-terminal residues that can be destabilizing in the cytosol and nucleus do not necessarily have the same effect in chloroplasts (83, 109).

N-terminomics: umbrella term for subproteomics; methods that specifically identify N termini of proteins from complex proteome samples

Cys-Initiated Proteins Are Rare

Cys is an infrequent N-terminal residue of plastid-encoded proteins, which otherwise show a comparable distribution of N termini. Together with Trp, Cys is the least abundant N terminus in the chloroplast-encoded proteome (1–2%). Moreover, Cys is highly enriched in the P1 position of the cleavage site of chloroplast transit peptides (cTPs); that is, cTP cleavage is likely to occur C-terminally of Cys but not anywhere else (34, 76, 109, 119). Chloroplast-localized RADIATION SENSITIVE 52-2 (RAD52-2, AT5G47870) is predicted to have a neoCys50 residue […VNRGVR|C40-SGGGVGGGDA…; the vertical bar (|) indicates the suggested protease cleavage site according to the identified following neo-N-terminal residue, which is indicated by the residue number of the full-length protein; the ellipsis (…) indicates the upstream and downstream residues] and has been proposed as a candidate substrate of *Arabidopsis* caseinolytic peptidase (Clp) S1, a conserved chloroplast substrate selector of the plastid ClpP protease system (23, 95). This adds up to significantly low abundance at the penultimate position of the N termini of nuclear-encoded proteins (approximately 240), which would theoretically expose neoCys after NME (**http://arabidopsis.org**). The description of the METACASPASE9 degradome led to an additional 25 cleaved protein fragments with a Cys at the neo–N terminus (117). Whether or not these can be targeted by the N-degron pathway remains to be seen.

N-terminomics identified Cys-initiated internal peptides of two *Arabidopsis* proteins: the plastid-encoded Rubisco large subunit (rbcL, ATCG00490) and the nuclear-encoded, cytosolic Tyr aminotransferase Cys lyase CORONATINE-INDUCED 1/JASMONIC ACID–RESPONSIVE 2 (CORI3, AT4G23600) (123). Neo-N-terminal Cys84 (…RYKGR|C84-YHIE…) was found for rbcL when using the three significantly different proteases, trypsin (cutting mainly after Arg and Lys), GluC (cutting mainly after Glu), and subtilisin (unspecific cleavage), in the proteomics workflow, indicating that the cleavage is naturally occurring. In the case of CORI3, only the sample treated with GluC revealed a neoCys6 (…MATLK|C6-IDWQ…), which might also represent a naturally occurring novel cleavage site (122).

Shotgun proteomics of N-degron pathway mutants, which gives no hint about the identity of the actual N terminus, revealed several diverse, upregulated Met-Cys-starting proteins in *ate1 ate2* and *prt6* mutants (86). Here, the arginylation branch appears to be interrupted. The abundance of two of the three cytosolic ASPARAGINE SYNTHETASEs was increased in the *ate1 ate2* double mutant but also the theoretically unrelated *prt1* single mutant. These proteins belong to the class of Met-Cys-starting proteins and most likely have N-terminal Cys residues after NME. Accumulation seen by mass spectrometry of *ate1 ate2* samples suggests possible recognition and modification by ATEs followed by subsequent ubiquitination of their Arg-conjugated form by PRT6; however, the Cys2 residues were shown to make up the catalytic site and to be required for activity. They are therefore unlikely to be surface exposed nor to function as pre-N-degrons.

Another N-terminomics study identified one Cys-initiated peptide with a neoCys293 (…ICSAR|C293-TDNLDDPSR|…) in *prt6* mutant seedlings (139). This was one out of 21 peptides of the vacuolar seed storage protein CRUCIFERIN 1 (CRU1, AT5G44120), which is

retained under normal conditions in storage compartments. Both N-terminal and C-terminal cleavage sites of the Cys-initiated peptide could be generated by tryptic digest as well.

NO scavenger: cPTIO counteracts NIA1/NIA2 and NOA1 function in vivo

Cys-Initiated Proteins in the Sensing of O_2 from the Air

In this context, stabilizing stress-related transcription factors transmit the hypoxic signal (45, 84, 88). To date, only four Met-Cys-starting ERFVIIs—RAP2.2, RAP2.12, HRE1, and HRE2—and one ERFVII unrelated protein—the Met-Cys-initiated VERNALIZATION2 (VRN2)—have been described as in vitro and in vivo N-degron pathway targets (45, 47). This is based on instability in in vitro rabbit reticulocyte lysate degradation experiments using hemagglutinin epitope-tagged protein versions. All five showed a significantly reduced half-life compared with variants where the N-terminal Met-Cys was substituted with Met-Ala. Ala is a stabilizing residue, at least in the Arg/N-degron pathway, and these substitutions are commonly used to test for the importance of the replaced native residue. In the cases of HRE1 and HRE2, addition to the assay of the proteasome inhibitor MG132 reduced degradation. Addition of Arg-Ala dipeptides that can inhibit UBR1 in mammalian and yeast systems (the N-recognin UBR1 is likely responsible for mediating degradation in rabbit reticulocyte lysate) led to slower degradation of the four ERFVIIs only insufficiently. Importantly, the rice ERFVII homolog SUBMERGENCE 1A (SUB1A) is stable in this assay, although it carries the highly conserved N-terminal amino acid sequence, including a Cys2, which is possibly oxidized as well (45). This suggests that the N-degron pathway became divergent in rice with respect to the group of ERFVII transcription factors as potential degradation targets.

RAP2.12, when N-terminally fused to GFP, is membrane-localized in normoxia and accumulates exclusively in the nucleus under hypoxia. The RAP2.12:GFP signal disappears after reoxygenation, suggesting an O_2-dependent degradation mechanism. This instability was not observed when the N terminus of RAP2.12 was truncated and lacked a short N-terminal sequence, including the initiator Met-Cys residues, or when it was analyzed in the background of *ate1 ate2* and *prt6* mutants. In the case of the latter, the still nuclearly localized signal was retained even under normoxic conditions. When using an antibody raised in rabbits against a synthetic peptide from the RAP2.12 sequence that has some similarity to RAP2.2 (N/KL/VKG/ASKKS/RSK/NN/KRSN/D), RAP2.12 was found to be stabilized under normoxia and reoxygenation in *ate1 ate2* and *prt6* mutants, as compared with the wild type, where lower levels were seen in air (84).

VRN2 is a developmentally crucial and well-studied component of the angiosperm polycomb repressive complex 2 (PRC2). During vernalization, which is the induction of flowering by exposure to prolonged cold, the VRN2-PRC2 complex methylates and thus silences the floral repressor gene *FLOWERING LOCUS C* (*FLC*). This process is significantly enhanced by cold-triggered accumulation of VRN2, but the molecular mechanism required to silence *FLC* is still unknown (134). Thus, VRN2 and related proteins encode a memory of cold that permits flowering once warm temperatures return. A recent paper showed that VRN2 was stabilized in vitro if the Met-Cys N terminus was substituted at position 2 with an Ala residue and, if expressed as a translational GUS fusion or tagged with HA or FLAG epitopes, in *ate1 ate2* as well as *prt6* mutants. VRN2-FLAG also accumulated if the proteasome inhibitor bortezomib was added to stably transformed plants or if introduced into the *prt6* mutant background. Then, VRN2-FLAG or VRN2-GUS accumulated in vivo if stably transformed *Arabidopsis* plants were submerged under water or exposed to the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) (47).

VRN2-GUS disappears from stably transformed plants after vernalization in the wild-type background but gets stabilized in *prt6*. Surprisingly, VRN2-GUS also disappears in the *prt6*

background where N-degron pathway–mediated proteolysis should be impaired, after 1 week of recovery at ambient temperature following vernalization (47). This seems to occur only if plants are returned to warm temperatures after being shifted from warm to cold treatment beforehand. In older plants (4 weeks) that have been vernalized or not, VRN2-GUS stays accumulated in *prt6* compared to wild type. Interestingly, HA-tagged RAP2.3 and HRE2, members of the previously characterized N-degron pathway substrate family of ERFVIIs, also accumulate after exposure to cold for 2 weeks. This correlated with increased transcription of hypoxic markers. Of note, both hypoxia and long-term cold exposure led to increased VRN2 abundance and elevated levels of VRN2-promoted tolerance to hypoxia by increasing levels of hypoxia-responsive genes (47).

NO donors: chemical donors for NO applications in vivo are SNAP, SNP, and GSNO

PRC2 complexes recruit various subunits, including FERTILIZATION INDEPENDENT 2 (FIS2) and EMBRYONIC FLOWER 2 (EMF2), in addition to VRN2. The Met-Cys N terminus of VRN2 homologs is highly conserved in angiosperms and in an EMF-related sequence partially between angiosperms and gymnosperms. The VRN2-like, Met-Cys-initiated barley (*Hordeum vulgare*) protein EMF2c (HvEMF2c), for example, can be stabilized in vitro if the N terminus is exchanged with an Ala residue or if a proteasome inhibitor is present (47). In *Arabidopsis*, the EMF2 N terminus contains 20 residues upstream of the highly conserved Met-Cys signature as in VRN2 and HvEMF2c. EMF2 was therefore suggested to contain an N-terminal cap shielding the conserved downstream Met-Cys residues; this is further supported by the fact that EMF2-HA is stable in cycloheximide chase assays of stably transformed *Arabidopsis*. In contrast, the artificially truncated (20 residues lacking) and Met-Cys-starting construct tEMF2-HA was instable under comparable conditions and required proteasomal activity. This effect could be fully abolished if the Cys were replaced by an Ala residue, as demonstrated for other Met-Cys-starting N-degron pathway substrates such as the ERFVIIs. Nevertheless, tEMF2-HA accumulated in *prt6* mutants and showed a similar cold-responsive accumulation after 2 weeks if vernalized as a stable transformant. Here, the same effect of increased degradation after returning the seedlings to ambient temperature could be observed as in the case of VRN2 (47). Whether this 20-amino-acid cap is removed in vivo remains speculative. However, posttranslational regulation of protein turnover of VRN2 and other components of PRC2 complexes potentially link environmental signals to the epigenetic control of plant development.

Cys-Initiated Proteins Sense Reactive Oxygen and Nitrogen Species

Another study showed clearly that HRE2 and RAP2.3 (EBP) were stabilized if cPTIO was added or if the proteins were isolated from submerged wild-type plants. If NO donors, such as the *S*-nitrosothiol *S*-nitroso-*N*-acetylpenicillamine (SNAP) or sodium nitroprusside (SNP) or *S*-nitrosoglutathione (GSNO), were added, the levels of HRE2 and RAP2.3 (EBP) were strongly reduced in the wild type. Moreover, HRE2 is stabilized in mutants for PRT6 and in NO-deficient mutants (*noa1*, *nia1 nia2*). NIA1 and NIA2 are the two functionally redundant nitrate reductases, and the NITRIC OXIDE-ASSOCIATED PROTEIN 1 (NOA1) functions with unknown mechanism (46). This mechanism regulates several developmental processes, including seed germination, stomatal closure, and hypocotyl elongation.

Two genetically encoded NO sensors were used in this study, Met-Cys-β-glucuronidase (MC-GUS) in *Arabidopsis* and MC-GGAIL-GUS in barley. Each sensor was compared with the corresponding stable Met-Ala-initiated controls. In contrast to classical genetically encoded biosensors (127), the ones used in the context of the N-degron pathway are either directly instable after cotranslational modification, such as Ub cleavage in Ub fusion constructs, or conditionally instable after N-terminal modification via NME, as in the case of Met-Cys-initiated reporters described above. These biosensors disappear from the cell by degradation.

N-terminally of the GUS reporter enzyme and following the two initiator residues Met and Cys, the MC-GUS contains an N-terminal oligo-Gly sequence, a part of *Escherichia coli* lacZ that is known as extension-containing Lys (eK), and a triple hemagglutinin tag (MC-RS-(G)12-RG-lacZ (eK)-3xHA-GUS). The eK sequence harbors two Lys residues, which were shown to act as Ub acceptors in many cases. The eK sequence was initially contained as a cloning scarf in the β-Gal test substrates, which led to the discovery of the so-called N-end rule (7). It is important to extract and be aware of the details of the reporter constructs because the amino acid residues and the sequence they make up are fundamental for reporter stability and have significant impacts on binding to N-degron pathway enzymes, turnover, and therefore readout (126). The reporter construct used in barley, MC-GGAIL-GUS, starts with the seven amino acids MCGGAIL, which are highly conserved among the ERFVIIs. The reporter construct therefore has—regardless of its longer name—a significantly shorter N-terminal extension preceding the GUS reporter enzyme compared with the MC-GUS. The MC-GUS is under control of the promoter for the 35S mRNA of *Cauliflower mosaic virus*, and MC-GGAIL-GUS is under the control of a Ub promoter.

ERFVIIs—especially RAP2.2, RAP2.3 (EBP), and RAP2.12—strongly induce the transcription of genes required for core anaerobic response, such as *HRA1*, *SAD6*, and *Hb1*. This effect was achieved by the transactivation of hypoxia-response promoters by N-terminal enhanced GFP (EGFP) fusions to the ERFVII sequences (EGFP:ERFVII). The effect was not as prominent for the other well-known hypoxia-responsive genes, *ADH*, *PDC1*, and *PCOs* (19). The three phytoglobins (plant hemoglobins) Hb1, Hb2, and Hb3 were shown only recently to have a strong tendency for autooxidation (91). Moreover, they act as NO scavengers via a NO-dioxygenase reaction and can bind O_2 (2, 98).

Interestingly, presence of a stable RAP2.3 (EBP) that was C-terminally fused to yellow fluorescent protein (YFP), YFP:RAP2.3, was seen to enhance dark-activated development and repress light-activated development to coordinate photomorphogenesis (1). Here, possible interaction of the N terminus of RAP2.3 with N-degron pathway components was literally blocked with the YFP moiety; however, if comparing YFP levels under normoxia versus hypoxia under light, the fusion seemed to accumulate slightly.

EXTENDING OUR KNOWLEDGE TO CROPS

The concept of N-degron pathway–mediated protein degradation of ERFVII transcription factors is considered to be a master O_2-sensing mechanism—if not the O_2-sensing mechanism—that mediates transitions between anaerobiosis under water stress and the normal aerobic growth of land plants, as needed (53). Important work was accomplished in barley as one of the world's key cereals and as a representative monocotyledon species. The barley ERFVII family member BERF1, which is most closely related to *Arabidopsis* RAP2.12, was shown to be an N-degron pathway target in vitro, and its highly conserved N-degron sequence was shown to be a portable N-degron that confers instability to a C-terminally-fused GUS enzyme as an artificial protein-stability reporter in vivo. Therefore, BERF1 and its N-terminal Cys residue are also considered to act as a sensor for oxygenation and flooding status in barley. Reducing the expression of the bona fide N-recognin E3 Ub ligase PRT6 by RNAi in barley increased the expression of hypoxia-related genes and generated an enhanced waterlogging tolerance (88). Transgenic barley with reduced *PRT6* expression showed enhanced resistance to waterlogging; abiotic stresses, such as salinity, drought, and high temperatures (124); and pathogens (125).

DISCOVERY OF NOVEL ENZYMATIC ROUTES

The insight that ERFVIIs function as O_2 sensors is based on the facts that these RAPs are stabilized in *ate* and *prt6* mutants and that Cys-starting proteins are instable in cell-free in vitro

degradation assays (45). There is recent molecular evidence for the existence of two of the three bona fide steps of the suggested metabolization cascade—Cys oxidation followed by N-terminal arginylation (132). However, subsequent binding to PRT6 as the predicted next enzyme of the N-degron pathway cascade and the polyubiquitination of ERFVIIs are still to be shown. The general underlying principle still comprises open questions; for example, in one study, N-degron pathway mutants seem to cope better with low O_2 (45); in another study, their coping was worse (84). This has been discussed in the literature and was likely caused by differences in the experimental setup (107, 110).

Biochemical Characterization of Cys Oxidation by PCOs Using Short Synthetic Peptides

Only recently have PCOs, the novel plant-specific class of enzymes, been associated with the N-degron pathway, and it is now known that the enzymatic oxidation pathway of Cys-starting proteins funnels them into the N-degron pathway metabolic cascade (130). The *PCO* gene family was discovered through a search for enzymes that could be involved in the oxidation of N-terminal Cys residues. Conserved among plants, PCOs include five members in *Arabidopsis*, PCO1 through PCO5. PCO1 and PCO2 are induced by hypoxia, repress the anaerobic response, and reduce tolerance to submergence. Short pentameric peptides of the conserved ERFVII sequence were used together with bacterially expressed PCO1 and PCO2 to test for the capability of Cys oxidation. First, sulphinic acid synthetase activity (Cys-oxidation activity) was measured by high-performance liquid chromatography (HPLC), using the free amino acid L-Cys and O_2 as substrates. The authors decided to use commercially available Cys sulphinic acid as a molecular standard for oxidized Cys to generate a calibration curve. All reactions also contained ascorbic acid as a reducing agent (69). The HPLC chromatograms of the PCO-containing Cys-oxidation reactions resembled the calibration standard of Cys sulfinic acid; however, at that point, it was not compared with fully oxidized Cys sulfonic acid, which appears to be more stable under many conditions. Cys sulfonic acid is also readily available as a standard chemical. The conclusion was that this oxidized form of Cys was also formed in both enzymatic reactions. It remains unclear whether only Cys sulfinic acid was produced under the reaction conditions or whether Cys sulfonic acid was produced as well. This could be tested in the future by simultaneous detection of both oxidation products of Cys in rat brain lysate with reasonable sensitivity and accuracy (68) or by simply using both chemicals as calibration standards.

In an O_2 consumption assay comparing free L-Cys, Cys-Ala dipeptides, and the native N-terminal ERFVII sequence CGGAI with various scrambled or control sequences, a second in vitro reaction showed that PCO1 and PCO2 could not oxidize internal Cys. O_2 consumption was shown to increase with the length of the substrate sequence. RAP2.12 was found to be stabilized in *pco1 pco2* mutants using the previously described antiRAP2.12 antibody (84), and a C-terminal fusion of Luc with the first 28 N-terminal amino acids of RAP2.12 (RAP1–28:Luc) was instable if either PCO1 or PCO2 was overexpressed (130).

The Missing Link: Cys Dioxygenation Enables N-Terminal Arginylation

Later, molecular enzymology of PCO1 and PCO4 showed that a partial Cys oxidation, the dioxygenation, is decisive for further PTM of Cys-initiated targets of the N-degron pathway. Moreover, for the first time, Arg transfer of *Arabidopsis* ATE1 could be demonstrated by mass spectrometry, ^{1}H-nuclear magnetic resonance spectroscopy, and biochemical assays (132). This transfer was only possible upon dioxygenation of the peptides that corresponded to the RAP2 ERFVII

transcription factors. Thereby, the work addressed speculations and uncertainties about the molecular link between PCOs, Cys oxidation, and ATE1 activity and function. The product of the PCO reaction was defined as N-terminal Cys sulfinic acid, rendering it a substrate for ATE1. A recent study has highlighted that the most catalytically competent PCO is PCO4 and has suggested its central role in ERFVII proteostasis regulation in hypoxia (131). This history effectively describes possible species-specific differences in enzymatic reactions and highlights the significance of the chosen experimental system in data interpretation.

All five PCOs were able to dioxygenate the 14-mer peptide CRQNCRAKSSPEEV corresponding to the N terminus of VRN2 after NME (47). This PTM occurred to a variable extent among all five PCOs. These oxidases also displayed temperature dependence (measured at 5°C or 21°C) in their dioxygenation capacity of N-terminal Cys from peptides (residues 2–15) corresponding to ERFVIIs and VRN2. This effect appeared to be unspecific for ERFVIIs in the cases of all PCOs. This is in strong contrast to PCO3, where the relative turnover of thiolated (Cys-containing) to dioxygenated ($CysO_2$-containing) peptides of VRN2 was strongly increased. Therefore, PCOs showed both a temperature- and a target-dependent specificity (47).

THE N-DEGRON PATHWAY HAS VARIOUS FUNCTIONS IN STRESS PHYSIOLOGY

A number of milestone papers have highlighted additional functions of the N-degron pathway in plants in response to stresses other than hypoxia caused by waterlogging or submergence. These roles span interactions with the biotic and abiotic environment.

The Cys/Arg Branch of the N-Degron Pathway in Response to Drought and Salinity

Stabilized ERFVIIs retard plant growth and are the molecular keys to the observed metabolic adjustments that increase tolerance to environmental stresses (124). This accumulation of ERFVIIs occurs in N-degron pathway mutants or under hypoxic conditions. The activity of nitrate reductase enhances their N-degron pathway–mediated degradation as it opposes ERFVII substrate stability via NO production. This was shown by using two artificial Cys-initiated substrates as in vivo NO reporters. The reporters were already utilized previously in *Arabidopsis* and barley as, respectively, MC-GUS with oligo-Gly and lacZ sequences (MC-HA-GUS) and MCGGAIL-GUS with the short N-terminal extension of seven ERFVII residues (46). To demonstrate the role of the Cys/Arg branch of the N-degron pathway, the precise involvement of ERFVII stability in abiotic stress response, apart from the known hypoxia-triggered effects, was established during various thermotolerance assays along with in vivo monitoring of NO and ROS levels. NO and ROS levels were additionally documented with readouts of salinity, drought, oxidative stress, and heat shock in both *Arabidopsis* and barley. These individual abiotic stresses were also tested in various combinations, and this revealed a novel role of the chromatin-remodeling ATPase BRAHMA (BRM) in plant response to salinity and ABA. BRM is a component of the switch/sucrose nonfermentable (SWI/SNF) nucleosome remodeling complexes, and this work demonstrated that the Cys/Arg branch of the N-degron pathway controls responses to multiple abiotic stresses via interaction between ERFVIIs and BRM.

In *Arabidopsis*, MC-GUS was instable in *nia1 nia2* mutants when pharmacologically complemented with the NO donor SNAP and in the wild type in the presence of high salt (NaCl) concentrations. MCGGAIL-GUS accumulated in barley when plants were watered with NaCl solution. These effects were detectable either by western blotting or histological GUS staining. Nitrate

reductase activity was reduced after drought in barley, leading to accumulation of the MCGGAIL-GUS signal in both western blots and GUS staining (124).

It was further suggested that reduced NO levels caused by reduced nitrate reductase activity interfere with the NO-sensing capability of the N-degron pathway. This in turn facilitates enhanced interaction between stress-stabilized ERFVIIs and BRM that is regulating downstream responses to enhance tolerance to salinity, for example. This mechanism provides a link between transcription factor function and chromatin remodeling and also represents a new concept of stress perception via targeted and conditional proteostasis of ERFVII transcription factors (124).

The Cys/Arg Branch of the N-Degron Pathway in Response to Pathogens

Arabidopsis with mutated *PRT6* had enhanced resistance against the virulent pathogen *Pseudomonas syringae* pv. *tomato* DC3000, and MC-HA-GUS levels were increased in these conditions and after injection with flg22. flg22 is a commonly used peptide-activating plant defense mechanism consisting of 22 amino acids of the conserved N-terminal part of pathogenic bacterial flagellin. flg22 was shown to not be able to enter compartments of the cell with N-degron pathway activity, such as the cytosol and nucleus; rather, it remained in the vascular tissue and was seen to enter the vacuole (70). In barley, RNAi was used to reduce transcript amounts of the PRT6 ortholog gene *HvPRT6*, and, followed by infection with *P. syringae* pv. *japonica* or *Blumeria graminis* f. sp. *hordei*, this resulted in accumulation of the artificial substrate MCGGAIL-GUS (125).

The role of the N-degron pathway and possibly regulated substrates is still under discussion, partially due to previously contradictory findings, where both *ate1 ate2* and *prt6* showed an increase in susceptibility to infection by various pathogens (25) and to clubroot root gall disease (58). Here, pathogen colonization after infection was tested with the necrotrophic fungi *Sclerotinia sclerotiorum* and *Botrytis cinerea*; the obligate biotrophic fungus *Erysiphe cruciferarum* (powdery mildew); and *Ralstonia solanacearum*, a soilborne bacterial pathogen; as well as *Pseudomonas syringae*, a model airborne bacterial pathogen for which both virulent (DC3000) and avirulent (AvrRpm1) strains exist. In all cases increased susceptibility for both *ate1 ate2* and *prt6* was described, except for the inoculation of *prt6* with DC3000, in which no deviation from the wild type could be seen (25). The root galls were induced by inoculation with the clubroot agent and protist *Plasmodiophora brassicae* (58).

However, the stabilization of Cys-initiated protein-stability reporters in both *Arabidopsis* and barley in response to infection has underlined that the involvement of the N-degron pathway is conserved in flowering plants and that the Gln-deamidation branch via NTAQ1 and the Cys-oxidation branch via ATEs are both components of the plant immune system through the E3 ligase and N-recognin PRT6 (125).

THE DISCOVERY OF POSTTRANSLATIONAL ARGINYLATION IN PLANTS

Whereas in animals, a handful of posttranslationally arginylated proteins can be detected by chemical Edman sequencing (24, 80) or by antibodies raised against peptides mimicking N-terminally arginylated target variants (20, 100), in the moss *P. patens* (Pp), targets of the moss Ate1 (111) were found by the immunoprecipitation of proteins with an antibody specific for N-terminally arginylated sequences (i.e., potentially modified by Arg transferases) (60) followed by mass spectrometry.

In one example from the plant kingdom, PpAARE, which presents for unknown reasons a neo-N-terminal Asp residue that was formerly Asp2 and that is initiated by Met, is an N-terminal arginylation that was found by mass spectrometry. Both the relevance and a potential function of

PpAARE N-terminal PTM by arginylation and its hypothetical link to the N-degron pathway–mediated degradation are still lacking (61). A few previously mentioned individual arginylation substrates from animals with relevance in autophagy have been discussed elsewhere (21, 73, 114, 116). In animals, N-terminal arginylation is increasingly considered to generate a bimodal degron, which operates both in autophagic and proteasomal proteolysis (136). No other in vivo arginylation targets that follow the N-degron pathway and massively degrade after arginylation have been identified.

THE FUTURE OF THE N-DEGRON PATHWAY: CONCLUSIONS

Foreseen: Applications Based on the N-Degron Pathway

Several studies dealt with interference to the N-degron pathway function in barley. The increased tolerance to a number of biotic and abiotic stresses that resulted indicates the possibility of translating these findings into crops. Degradation of ERFVIIs and the physiological, including agronomical, implications of this degradation have been intensively discussed in the literature. Possible future directions for this research include pharmacological modulation of the N-degron pathway to increase and decrease turnover of substrates. This would include the use of portable degradation tags (N-degrons), which are regulated by small molecules, by environmental cues such as temperature (26, 35–38), or by inhibitory peptides and peptidomimetic substances. The last would bind to the recognition domains of the N-degron pathway enzymes and interfere with the amino-terminal modification of such proteins and their subsequent recognition by E3s and related recognition elements of the N-degron pathway (10, 71, 78).

Needed: Approaches for the Identification of N-Degron Pathway Substrates

Several proteomic workflows have been used in the past to discover N-degron pathway targets in plants based on shotgun proteomics (86). These include N-terminomics (29, 99, 109, 122, 138), immunoprecipitation-assisted proteomics (61, 99), and immunological detection of N-terminal PTMs (20, 60, 99, 100, 111), and they have been comprehensively reviewed elsewhere (99). Additional discovery of novel N-degron pathway substrates is needed to better understand the actual roles of the entire N-degron pathway in plants. Future challenges include the definite detection of proteolytic sites, such as identifying P1′ residues downstream of the cutting site, and precise characterization of N-terminal PTMs. Artefacts generated by mass spectrometric or chemical procedures have clearly been flagged in the past as major pitfalls in N-degron pathway substrate identification and have often precluded a precise chemical characterization of the relevant PTMs. Special emphasis must be placed on the minor or difficult-to-see PTMs, namely deamidation by NTAs, Cys oxidation, and arginylation. In this context, use of nonstandard proteases, such as LysargiNase, that do not interfere with possible N-terminal PTMs (e.g., Arg transfer to the N terminus) has been suggested (66, 99). The detection and characterization of N-terminal Cys oxidation is very challenging, but several fluorescent and affinity-based tools are available to detect Cys sulfenic acid formation in proteins (101, 102).

In line with the rather technical and chemical protocols mentioned previously, the array of methods available for identification of novel N-degron pathway substrates should be more frequently used across proteomics studies, in vitro and in vivo investigations, genetics, and physiology.

The identification and biological role for PTMs in plants, in particular, are just emerging, yet what is known has been based predominantly on genetic observations. One of the main uncertainties in the field of targeted proteolysis via the N-degron pathway concerns the current

state-of-the-art understanding; that is, the real evidence-based characterization of molecular events that researchers have documented by their data. Several molecular steps within the enzymatic N-degron pathway reaction cascade are still obscure, including how the PTM of target proteins occurs especially—but not exclusively—at their N termini. There is no rigorous chemical validation of PTMs—especially for the arginylation branch. This is also true for the plant field: important biological functions of the N-degron pathway have been described, but the molecular basis for these functions is still largely lacking. In the process of discovering novel substrates and biological functions of the N-degron pathway, it is likely that many nontargets will be identified, and the involvement of the N-degron pathway in determining the stability of a protein may be unclear. Therefore, I strongly encourage publication of so-called negative but otherwise confirmatory study results in which the role of the N-degron pathway remains elusive. As Alex Varshavsky recalled in 1986 in a commentary on the first "N-end rule" paper, "It was clear that the system would be horribly complex" (77, p. 151).

SUMMARY POINTS

1. One principle of the N-degron pathway conserved throughout the biological kingdoms is its capacity for potential destabilization of a protein depending on the protein's N-terminal amino acid and the follow-up sequence. Different "N-end rules," or N-degron pathways, with variable mechanisms have been identified in yeast, bacteria, animals, and plants.
2. Every single N-terminal amino acid can lead to destabilization according to one of the described N-degron pathways. This is true for the classical destabilizing residues as well as for the classical stabilizing residues, including Met, Ala, Gly, Val, Ser, and Thr. The mere presence of N-terminal destabilizing residues is not sufficient to render a protein a target of the N-degron pathway. Final degradation strongly depends on the subcellular localization and posttranslational modifications (PTMs).
3. The core N-degron pathway machinery is conserved among animals, yeast, and plants. The plant components are encoded by multiple genes or represent plant-specific enzymes, suggesting diversification, subfunctionalization, and neofunctionalization of enzymatic components.
4. Plants, yeast, and animals mostly utilize the ubiquitin proteasome system to degrade N-degron pathway targets. Bacteria address substrate recognition via proteases and their adapters. In animals, arginylation is directly linked to target neofunctionalization and autophagy.
5. Endoproteases and aminopeptidases are needed to prime preproproteins with dormant or incompletely modified future N-degrons to make these available for the N-degron pathway biochemical reaction cascade.
6. N-degron pathway mutants are not compromised under normal conditions and are available for several plant species, including barley, moss, and in the background of various *Arabidopsis* accessions. They permit in-depth studies on the role of the N-degron pathway in plant growth, development, and survival under abiotic and biotic stresses.
7. Expression of N-degron pathway components appears to be constitutive and ubiquitous, suggesting that proteins may act on standby as a collective quality control system across the entire plant, in all organs and all cell types.

8. Enzymatic and nonenzymatic N-terminal Cys oxidation is an emerging subdivision of the arginylation branch of the Arg/N-degron pathway in animals and of the PRT6 N-degron pathway in plants. It involves a series of events, including N-terminal arginylation, which can transfer a destabilizing amino acid and generate an N-degron.
9. Only very recently have the biochemistry and molecular mechanisms of Cys oxidation coupled to N-terminal arginylation been described. The substrates harboring the characterized N terminus, which serves as acceptor of these PTMs, belong to the highly conserved transcription factors of group VII of the B-2 subfamily of ETHYLENE RESPONSE FACTORs (ERFVIIs).
10. Applications of modulated environmental stress response are currently entering crop breeding. The first crop in which N-degron pathway mutants were investigated and shown to demonstrate beneficial effects was barley.

FUTURE ISSUES

1. What are the unknown unknowns of the N-degron pathway regarding substrates and biological functions of targeted protein degradation or neofunctionalization?
2. How can we better identify and characterize novel substrates?
3. What are the gold standards to discriminate substrates versus nonsubstrates and to reduce artefacts and the incomplete characterization of targets?
4. What is the function of non-Cys/Arg branch N-degron pathway targets, and how many are there?
5. How can we best identify substrates that are generated conditionally and only present transiently in the cell?
6. Is substrate generation via proteases triggered by environmental or developmental cues?
7. If there is a chloroplast N-degron pathway, what is it, and which enzymes and interactors are involved in the degradation of targets?
8. The N-degron pathway can be inhibited by short peptides and peptidomimetic substances in yeast and mammalian cell culture. Does this work in plants, and can this be used to beneficially manipulate protein turnover?

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