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Structure and evolutionary conservation of the plant N-end rule pathway

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SUMMARY

The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal amino acid residue. While some N-terminal residues result in metabolically stable proteins, other, so-called destabilizing residues, lead to rapid protein turnover. The N-end rule pathway, which mediates the recognition and degradation of proteins with N-terminal destabilizing residues, is present in all organisms examined, including prokaryotes. This protein degradation pathway has a hierarchical organization in which some N-terminal residues, called primary destabilizing residues, are directly recognized by specific ubiquitin ligases. Other destabilizing residues, termed secondary and tertiary destabilizing residues, require modifications before the corresponding proteins can be targeted for degradation by ubiquitin ligases. In eukaryotes, the N-end rule pathway is a part of the ubiquitin/proteasome system and is known to play essential roles in a broad range of biological processes in fungi, animals and plants. While the structure of the N-end rule pathway has been extensively studied in yeast and mammals, knowledge of its organization in plants is limited. Using both tobacco and Arabidopsis, we identified the complete sets destabilizing and stabilizing N-terminal residues. We also characterized the hierarchical organization of the plant N-end rule by identifying and determining the specificity of two distinct N-terminal amidohydrolases (Nt-amidases) of Arabidopsis that are essential for the destabilizing activity of the tertiary destabilizing residues Asn and Gln. Our results indicate that both the N-end rule itself and mechanistic aspects of the N-end rule pathway in angiosperms are very similar to those of mammals.

Keywords: protein degradation, N-end rule, Nt-amidase, R-transferase.

INTRODUCTION

In eukaryotes, the control of protein stability is carried out to a large extent by the ubiquitin–proteasome system, which mediates the conjugation of an 8-kDa protein called ubiquitin (Ub) to target proteins, marking them for proteolysis. Ub is conjugated to lysine residues of substrate proteins through the action of three enzymes, E1, E2 and E3 (Hershko *et al.*, 2000; Varshavsky, 2006). The selectivity of ubiquitylation is mediated primarily by E3 Ub ligases, which recognize specific degradation signals (degrons) of substrate proteins. A ubiquitylated protein bears a covalently linked poly-Ub chain and is targeted for degradation by the 26S proteasome (Hanna and Finley, 2007). Regulated proteolysis by the Ub system underlies about every significant cellular and organismal function in eukaryotes. In plants, Ub-dependent processes play major and diverse roles, for example in regulating the signaling by phytohormones such as auxin, gibberellins and jasmonic acid (Moon *et al.*, 2004; Bishopp *et al.*, 2006; Vierstra, 2009 and references therein).

An essential determinant of one class of degrons, called N-degrons, is a substrate's destabilizing N-terminal residue. The set of destabilizing residues in an organism yields a rule, called the N-end rule, which relates the *in vivo* half-life of a protein to the identity of its N-terminal residue (Figure 1) (Bachmair *et al.*, 1986; Varshavsky, 1996; Hu *et al.*, 2005; Tasaki and Kwon, 2007). In eukaryotes, the N-degron consists of three determinants: a destabilizing N-terminal residue of a protein substrate, its internal Lys residue(s), and a conformationally flexible region(s) in the vicinity of these determinants (Bachmair and Varshavsky, 1989).

The N-end rule has a hierarchical structure (Figure 1). In fungi and animals, N-terminal Asn and Gln are tertiary destabilizing residues in that they function through their enzymatic deamidation, to yield the secondary destabilizing N-terminal residues Asp and Glu. In the yeast *Saccharomyces cerevisiae*, the N-terminal amidohydrolase (noted Nt-amidase) NTA1 can deamidate both N-terminal Asn and N-terminal Gln (Baker and Varshavsky, 1995) (Figure 1a).

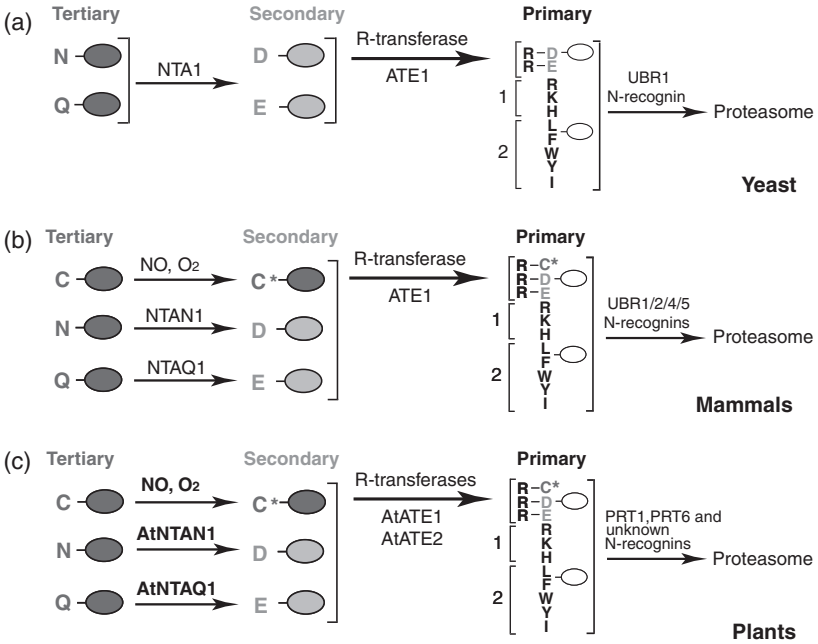

Figure 1. Schematic representation of the N-end rule pathway in yeast, mammals and plants. Ovals denote a protein substrate. N-terminal residues are indicated by single-letter abbreviations. C* denotes oxidized N-terminal Cys.
(a) In the yeast *Saccharomyces cerevisiae*, tertiary destabilizing residues (Asn and Gln) are deamidated by a single Nt-amidase (NTA1), while the R-transferase ATE1 recognizes proteins with the secondary destabilizing residues Asp and Glu, and conjugates Arg to their N-termini. The yeast genome encodes only one N-recognin, UBR1, which binds to type 1 (basic) and type 2 (bulky hydrophobic) residues (Varshavsky, 1996) and references therein).
(b) The N-end rule pathway in mammals is overall similar to that of yeast, but exhibits certain differences. For example, two distinct Nt-amidases (NTAN1 and NTAQ1) are involved in the deamidation of N-terminal Asn and Gln, respectively (Kwon *et al.*, 2000; Wang *et al.*, 2009). In mammals, Cys is an additional tertiary destabilizing residue, which requires oxidation through a chemical reaction involving NO and oxygen, prior to its recognition by isoforms of the R-transferase ATE1 (Hu *et al.*, 2005; Lee *et al.*, 2005). Contrary to yeast, the genomes of mammals encode several N-recognins (UBR1, 2, 4 and 5) (Tasaki *et al.*, 2005).
(c) The hierarchical organization of the N-end rule pathway in plants is similar to that found in mammals (this study). Only 2 N-recognins, PRT1 and PRT6, have been identified to date, but additional N-recognins are likely present.

In contrast, animals have two distinct Nt-amidases, an Asn-specific Nt-amidase (Nt^N-amidase or NTAN1) (Grigoryev *et al.*, 1996; Kwon *et al.*, 2000) and a recently discovered Gln-specific Nt-amidase, termed Nt^Q-amidase or NTAQ1 (Wang *et al.*, 2009) (Figure 1b). The activity of the secondary destabilizing N-terminal residues Asp and Glu requires their conjugation, by the Arg–tRNA–protein transferase (noted R-transferase), to Arg, one of the primary destabilizing residues (Varshavsky, 1996; Kwon *et al.*, 2002; Hu *et al.*, 2006). In mammals, the set of arginylated residues also comprises N-terminal Cys, which is arginylated *in vivo* after its non-enzymatic oxidation, in a reaction that involves nitric oxide (NO) and oxygen (Hu *et al.*, 2005; Lee *et al.*, 2005) (Figure 1b). Whereas a single gene, *ATE1*, encodes R-transferase in *S. cerevisiae* and the mouse or human genomes (Kwon *et al.*, 1999, 2002), Arabidopsis contains two closely related genes, *AtATE1* (*At5g05700*) and *AtATE2* (*At3g11240*) (Yoshida *et al.*, 2002) (Figure 1c). Primary destabilizing residues are recognized by E3 Ub ligases of the N-end rule pathway, termed N-recognins (Varshavsky, 1996; Tasaki *et al.*, 2005, 2009). While a single N-recognin is present in *S. cerevisiae* (Xia *et al.*, 2008) (Figure 1a), mammalian genomes encode at least four distinct N-recognins (Tasaki *et al.*, 2005, 2009), which contain a conserved domain termed UBR domain (Tasaki *et al.*, 2005). In Arabidopsis, two N-recognins, PROTEOLYSIS 1 (PRT1) and PRT6, have been identified (Bachmair *et al.*, 1993; Potuschak *et al.*, 1998; Stary *et al.*, 2003; Garzon *et al.*, 2007) (Figure 1c). PRT1 was uncovered in a genetic screen that aimed at identifying genes involved in the degradation of N-end rule substrates bearing Phe at their N-terminus (Bachmair *et al.*, 1993) and analysis of its sequence indicates that it does not have strong similarities to other known N-recognins (Stary *et al.*, 2003). In contrast, PRT6 was identified based on its sequence similarities to UBR1, and also presents the characteristic UBR domain (Garzon *et al.*, 2007). Experimental evidence and sequence similarity searches using mammalian N-recognins as queries suggest that other N-recognins are likely to be present in plants (Worley *et al.*, 1998; Stary *et al.*, 2003; Tasaki *et al.*, 2005; Garzon *et al.*, 2007). For example, BLASTP searches using mammalian UBR4 as a query led to the identification of BIG (Tasaki *et al.*, 2005) (also known as TIR3 or DOC1) as a candidate N-recognin.

Whereas in animals and fungi the N-end rule pathway is known to mediate the control of diverse cellular and developmental processes ((Tasaki and Kwon, 2007;

Varshavsky, 1996) and references therein), its functions in plants are only beginning to emerge. Yoshida *et al.* (2002) demonstrated that the R-transferase-coding gene *AtATE1* is disrupted in the Arabidopsis mutant *delayed leaf senescence1* (*dls1*), in which both age-dependent and dark-induced leaf senescence are abnormally slow. Recently, it was shown that the N-recognin PRT6 and the R-transferases AtATE1 and AtATE2 are involved in promoting seed germination and establishment through the removal of sensitivity to the hormone abscisic acid (Holman *et al.*, 2009), as well as in the regulation of leaf morphology, apical dominance and stem elongation (Graciet *et al.*, 2009).

Despite the emerging evidence for an involvement of the N-end rule pathway in the control of plant development, the functional understanding of this pathway is currently limited by lack of *bona fide* substrates. In addition, knowledge about the structure and hierarchical organization of the plant N-end rule pathway, which would be essential for a systematic identification of its substrates, is incomplete. For example, the sets of stabilizing and destabilizing residues of this pathway are defined only in part (Worley *et al.*, 1998; Yoshida *et al.*, 2002; Stary *et al.*, 2003). In the present study, we have systematically dissected the structure of the plant N-end rule pathway and have identified two Arabidopsis Nt-amidases, which are required for the recognition of tertiary destabilizing residues. Our results show that the plant N-end rule pathway is similar to that of animals and we discuss the implications that this finding has on the evolutionary history of this essential protein degradation pathway.

RESULTS

Identification of stabilizing and destabilizing N-terminal residues in tobacco

Methionine aminopeptidases (MetAPs) remove Met from the N-terminus of a newly formed protein only if the residue at position 2, to be made N-terminal after cleavage, has a small enough side chain (Huang *et al.*, 1987; Bradshaw *et al.*, 1998). Consequently, amongst the 13 destabilizing residues of the mammalian N-end rule (Figure 1b), only Cys can be made N-terminal by MetAPs. However, any destabilizing residue can be made N-terminal through internal cleavages of proteins by other proteases, such as separases, caspases and calpains (Varshavsky, 1996; Ditzel *et al.*, 2003).

Previously identified destabilizing residues in the plant N-end rule were identified through the Ub fusion technique (Bachmair *et al.*, 1986; Varshavsky, 2005) and sets of engineered N-end rule substrates, such as those based on dihydrofolate reductase (DHFR) (Bachmair *et al.*, 1993; Potuschak *et al.*, 1998), β-glucuronidase (GUS) (Worley *et al.*, 1998; Garzon *et al.*, 2007), or luciferase (LUC) (Worley *et al.*, 1998). These proteins, engineered as fusions to an N-terminal Ub moiety (e.g. Ub–X–LUC), are co-translationally deubiquitylated *in vivo* (Turner and Varshavsky, 2000), yielding otherwise identical reporter proteins with different N-terminal residues X (Figure 2a). The *in vivo* degradation of these reporters can be assessed either by pulse–chase assays or by measuring steady-state levels of the X-reporter proteins, and comparing the resulting values to the level of an otherwise identical reporter protein with a stabilizing

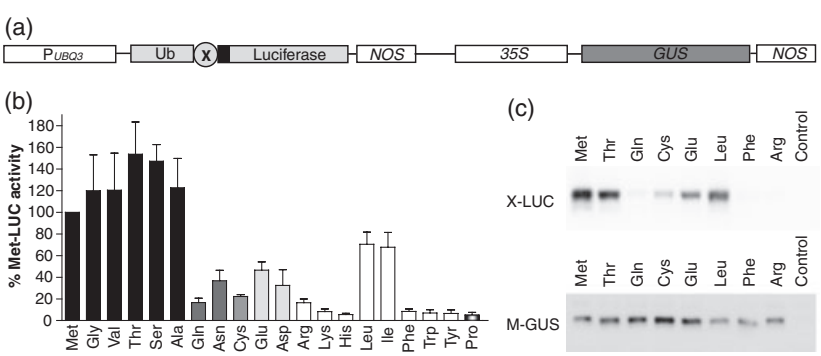

Figure 2. Characterization of the N-end rule pathway in tobacco.
(a) Ubiquitin fusion reporter constructs. The constructs used to determine the effects of different N-terminal residues on protein stability encoded a Ub–X–LUC reporter with varying residues at position X (Worley *et al.*, 1998). A short linker (represented by a black rectangle) is present between the varying residue X and LUC. Ub–X–LUC reporters were expressed from the ubiquitin *UBQ3* promoter, while the GUS-based 'reference' protein was expressed from the CaMV 35S promoter (Worley *et al.*, 1998). NOS: nopaline synthase transcriptional terminator sequence.
(b) Relative metabolic stabilities of X–LUC reporters in tobacco. LUC activities were normalized against the corresponding GUS activities and compared with Met–LUC activity, which was set to 100%. Black bars indicate stabilizing residues, while white bars denote primary destabilizing residues. Tertiary and secondary destabilizing residues are represented by dark and light-grey bars, respectively. Note that Pro is likely not a destabilizing residue. Error bars represent standard errors calculated from four independent experiments, except for Leu, Ile, Gly and Val, for which six data points were produced.
(c) Western blot analysis of GUS and LUC protein levels. Protein extracts representing equal amounts of GUS activity (1815 nmol min^{-1} μg^{-1}) were separated by SDS–PAGE for selected X–LUC reporters. Western blot analysis was carried out using GUS- or LUC-specific antibodies to confirm the presence of approximately equal amounts of GUS protein in the samples (lower panel) and to determine the levels of LUC protein (upper panel).

N-terminal residue (e.g. Met) (Bachmair *et al.*, 1986; Bachmair and Varshavsky, 1989; Varshavsky, 2005). Previous work showed that this 'steady-state' approach is a sensitive method to compare metabolic stabilities of proteins, especially of those that differ solely at the first-residue position (Bachmair and Varshavsky, 1989; Varshavsky, 2005).

To determine the full complement of stabilizing and destabilizing residues in plants, we employed an N-end rule reporter described by Worley *et al.* (1998), which consist of Ub fusions to firefly luciferase (LUC), with a varying junctional residue X that becomes N-terminal upon *in vivo* deubiquitylation (Figure S1). The N-end rule reporter used also encodes for a linker between the varying residue X and LUC, which resulted in the addition of a Lys residue that can serve as ubiquitylation site (Worley *et al.*, 1998) (Figure 2a). These reporters also allow the expression of a long-lived 'reference' protein (GUS), which is used to normalize LUC protein levels or activities for differences in transformation efficiencies or expression levels (Figure 2a). Using the construct from Worley *et al.* we produced a set of 20 binary vectors for expression of X–LUC reporters with all possible N-terminal amino acid residues. These plasmids were employed for agroinfiltration of tobacco leaves (Yang *et al.*, 2000; Wroblewski *et al.*, 2005). Following agroinfiltration, the activities of GUS and LUC were measured in leaf extracts.

The correlation between the levels of a given X–LUC reporter protein and its metabolic stability relies on the assumption that the measurements are carried out at the steady state (Varshavsky, 2005). In transient expression experiments, steady state may be reached at different time points after agroinfiltration, depending on the nature (stabilizing or destabilizing) of the N-terminal residue. In particular, it was possible that the steady state of X–LUC reporter proteins bearing stabilizing N-terminal residues would be reached after that of reporters starting with destabilizing residues. To test this possibility, we measured the activities of GUS and LUC at different time points after agroinfiltration and found that the steady-state levels of these proteins were typically established between 24 and 48 h post-infiltration (data not shown). However, for X–LUC reporters starting with a stabilizing residue such as Met, steady state was sometimes not reached before the LUC activity would begin to decrease (at about 3 days post-infiltration), possibly introducing variability into our assays.

Figure 2(b) shows the relative levels of X–LUC reporters (normalized against the levels of GUS, with the level of Met–LUC set to 100%) as a function of their N-terminal residues. N-terminal residues that conferred a level of X–LUC lower than 75% that of Met–LUC were denoted as 'destabilizing' residues, with the rest classified as 'stabilizing'. By these criteria, N-terminal Met, Gly, Val, Thr, Ser, and Ala are stabilizing residues in plants, whereas N-terminal Gln, Asn, Cys, Glu, Asp, Arg, Lys, His, Leu, Ile, Phe, Trp, and Tyr are destabilizing residues (Figure 2b). Although Pro–LUC activity was very low, Pro is unlikely to be a destabilizing residue. Previous work has shown that deubiquitinating enzymes (DUBs) cleave the Ub–Pro peptide bond more slowly than the analogous peptide bonds between Ub and another downstream amino acid residue (Bachmair *et al.*, 1986). A Ub fusion in which the N-terminal Ub moiety is either non-removable by DUBs (e.g. through an alteration of the last residue of Ub) or is removed slowly (as in the case of the Ub–Pro bond) is targeted for degradation by a distinct (non-N-end rule) pathway of the Ub system, termed the UFD (Ub–fusion–degradation) pathway (Johnson *et al.*, 1992; Hwang *et al.*, 2009). The UFD pathway is likely to be present in plants as well, as Worley *et al.* have shown that expression of a Ub*–Met–LUC fusion, in which the last four residues of Ub have been removed (noted Ub*) is unstable compared with its Met–LUC equivalent (Worley *et al.*, 1998). In good agreement with this possibility, the Ub–Pro–LUC fusion did not accumulate to detectable levels (Figure S1). Thus, the low level of LUC activity from the Ub–Pro–LUC reporter (Figure 2b) likely results from its targeting for degradation by the UFD pathway, as distinguished from the N-end rule pathway or from a loss of LUC activity due to absence of deubiquitylation.

To confirm that the differences in LUC activity were indeed the result of different LUC protein levels and were not caused by changes in the enzymatic properties of either LUC or GUS in the protein extracts tested, we carried out western blot analysis using GUS and LUC-specific antibodies (Figures 2c and S1). When protein samples with equal GUS activities were loaded, similar levels of GUS protein were detected in the different samples (Figure 2c). In contrast, the levels of X–LUC reporter proteins varied (Figures 2c and S1) and reflected approximately the relative LUC activities calculated from the quantitative enzymatic assays (Figure 2b). These results indicate that the differences in X–LUC activities correlate with variations in protein levels and thus depend on the nature of the N-terminal residue X.

Conservation of the N-end rule between tobacco and Arabidopsis

In order to validate the results of the agroinfiltration experiments, we generated, in Arabidopsis, stable transformants for a subset of N-end rule reporter constructs representing both putative stabilizing and destabilizing residues. We measured GUS and X–LUC activities in these lines and then normalized the data obtained for X–LUC using GUS activities to compensate for differences in expression levels. For the set of destabilizing N-terminal residues tested, we found that relative X–LUC activities (when compared with Met–LUC) were similar to those observed in tobacco (Figure 3a). Furthermore, western blot analysis using GUS and LUC-specific antibodies showed that the GUS and LUC enzymatic activities correlated with the levels of the corresponding

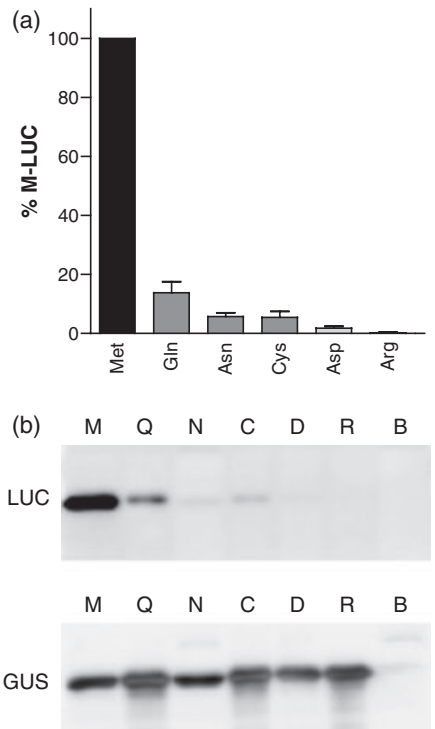

Figure 3. Characterization of Arabidopsis plants stably transformed with selected N-end rule reporters. Results from representative lines are shown.
(a) Relative X–LUC activities. LUC activities were normalized against the corresponding GUS activities, and compared with that of Met–LUC, whose activity was set to 100%. Error bars represent standard errors calculated from 3 independent experiments.
(b) GUS and LUC enzymatic activities correlate with protein levels. Protein extracts representing equal amounts of GUS activity (25 150 nmol min^{-1} μg^{-1}) were separated by SDS–PAGE. Western blot analysis was carried out to confirm the presence of approximately equal amounts of GUS protein in the samples (lower panel) and to determine the levels of LUC protein (upper panel).

proteins (Figure 3b). Together, these results confirm the validity of the agroinfiltration experiments described above.

To directly demonstrate that the low levels of X–LUC reporter proteins with N-terminal destabilizing residues were due to a short half-life, we attempted cell-free degradation assays using protein extracts from wild-type Arabidopsis seedlings and a purified Ub–Arg–LUC fusion. Arg–LUC was chosen as a reporter because Arg is a primary destabilizing residue that is likely to result in a very short half-life. This should lead to a relatively rapid decrease of the reporter protein when added to a protein extract containing active N-recognins and proteasome. Although the Ub–Arg–LUC fusion was efficiently deubiquitylated in the protein extract in the presence of the exogenously added deubiquitinating enzyme USP2 (Catanzariti *et al.*, 2004), degradation of Arg–LUC was not observed (data not shown), presumably because the extraction or assay conditions resulted in inactivation of the N-recognins. We also attempted cycloheximide chases on seedlings and protoplasts from stable transformants expressing the Ub–Arg–LUC fusion. Addition of the translational inhibitor cycloheximide should lead to a rapid decrease of the reporter protein. However, the Arg–LUC reporter did not accumulate to detectable levels, even after pre-incubation of the seedlings or protoplasts with MG132, a reversible inhibitor of the 26S proteasome (data not shown). This precluded the use of cycloheximide (or pulse) chases to directly show that the different levels of X–LUC reporter proteins are related to differential metabolic stabilities, an experimental limitation that had also been noted in previous studies (Potuschak *et al.*, 1998; Worley *et al.*, 1998).

In summary, the results obtained using stable N-end rule reporter lines confirm the data obtained using transient expression assays and further suggest that the sets of stabilizing and destabilizing residues are conserved between tobacco and Arabidopsis.

Identification of Arabidopsis Nt^N- and Nt^Q-amidases

Previous work has shown that N-terminal Asp and Glu are secondary destabilizing residues in Arabidopsis (Yoshida *et al.*, 2002; Graciet *et al.*, 2009) (Figure 1). Proteins bearing such N-terminal residues require conjugation to the primary destabilizing residue Arg by Arg-transferases, before they can be recognized by N-recognins and targeted for degradation by the N-end rule pathway. The conservation of the identity of the secondary destabilizing residues and of Arg-transferases in Arabidopsis implies that the plant N-end rule has a hierarchical organization similar to that of the N-end rule in fungi and mammals (Figure 1) (Varshavsky, 1996; Hu *et al.*, 2005; Lee *et al.*, 2005). This idea was further supported by our finding that reporters bearing N-terminal Gln or Asn, which are tertiary destabilizing residues in yeast and mammals, were short-lived in plants (Figures 2 and 3). Protein substrates starting with Gln or Asn are known to require two sequential modifications, which involve two classes of enzymes, Nt-amidases and Arg-transferases (see Introduction), before they can be targeted for degradation by the N-end rule pathway.

We reasoned that if Asn and Gln were indeed tertiary destabilizing residues in plants, then Asn–LUC and Gln–LUC reporter proteins should become stable when expressed in an Arabidopsis mutant lacking functional R-transferases (*ate1-2 ate2-1*, noted *ate1 ate2* hereafter) (Graciet *et al.*, 2009; Holman *et al.*, 2009). The Arabidopsis R-transferases AtATE1 and AtATE2 have been shown to conjugate Arg to protein substrates bearing N-terminal Asp or Glu (Yoshida *et al.*, 2002; Graciet *et al.*, 2009), and should therefore be required for the destabilization of Asn–LUC and Gln–LUC. To test this hypothesis, we transformed *ate1 ate2* mutant plants with a subset of X–LUC N-end rule reporters and carried out western blot analysis using representative *ate1 ate2* N-end rule reporter lines from the T3 generation. The results of the western blot analysis showed that the levels of Gln–LUC and

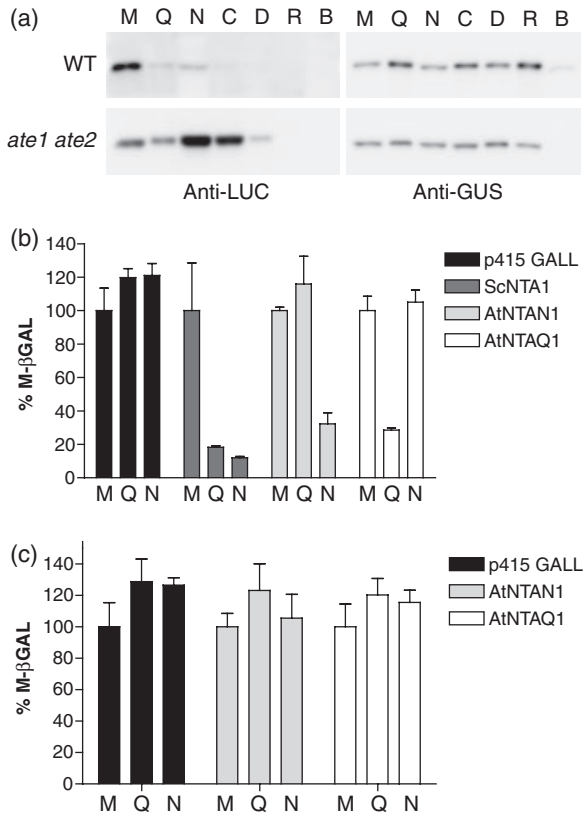

Figure 4. Identification of the plant Nt^N- and Nt^Q-amidases.
(a) Stabilization of N-end rule reporters bearing tertiary and secondary destabilizing residues in *ate1 ate2* double-mutant plants. Wild-type (upper panels) and *ate1 ate2* double-mutant (lower panels) plants stably transformed with selected Ub–X–LUC N-end rule reporter constructs were isolated and characterized. After measuring GUS and LUC activities, protein extracts representing equal amounts of GUS activities (equivalent to 10 000 nmol min^{-1} μg^{-1}) were separated by SDS–PAGE. Western blot analysis was carried out to confirm the presence of approximately equal amounts of GUS protein in the samples (right panel) and to determine the levels of LUC protein (left panel).
(b) Expression of *At2g44420* (putative *AtNTAN1*) and *At2g41760* (putative *AtNTAQ1*) in a yeast *nta1Δ* mutant restores degradation of Asn-βGal and Gln-βGal, respectively. βGal activities derived from p416 GALL:Ub–X–βGal reporters were measured in the presence of the expression vectors p415 *GALL* HA_6, p415 *GALL:ScNTA1-*HA_6, p415 *GALL:AtNTAN1-*HA_6, or p415 *GALL:AtNTAQ1-*HA_6. The βGal activities for different N-terminal residues are shown relative to those for Met–βGal. Error bars represent standard errors calculated from 3 independent experiments.
(c) R-transferase activity is required for the destabilization of Asn-βGal and Gln-βGal by AtNTAN1 and AtNTAQ1 in yeast. The experiment was carried out as described in (b), except that a yeast *ate1Δ nta1Δ* double mutant was used. Error bars represent standard errors calculated from three independent experiments.

Asn–LUC were considerably higher in *ate1 ate2* plants compared with the wild type (Figure 4a), suggesting that these two reporter constructs were stabilized in the *ate1 ate2* double-mutant background. While these results were in agreement with Asn and Gln being tertiary destabilizing residues in Arabidopsis, Nt-amidases, which are required for the recognition and processing of such substrates, had not been described in plants.

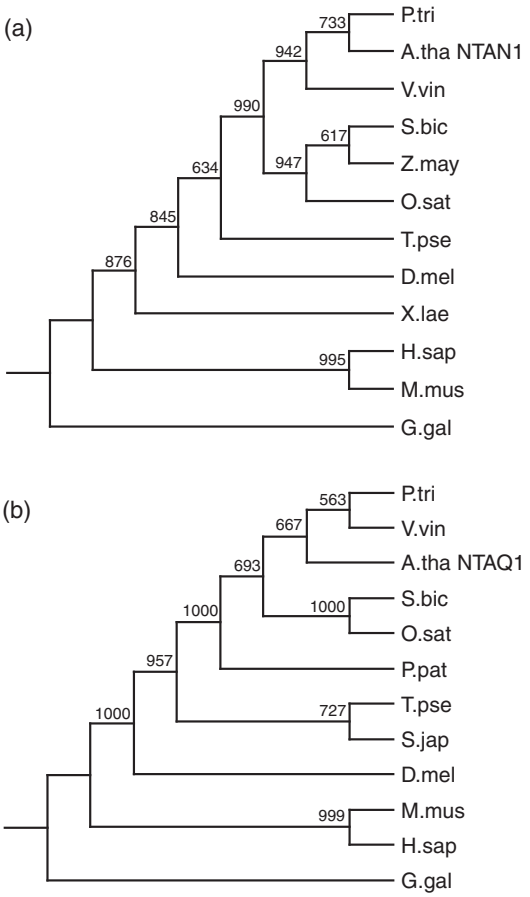

Figure 5. Protein distance-based phylogenetic analysis of selected NTAN1 and NTAQ1 orthologues.
(a) NTAN1 phylogenetic analysis. Sequences were retrieved using BLASTP with mouse NTAN1 as a query. Numbers by phylogenetic branch points give their statistical strength, with 1000 being the highest score. Complete names of indicated organisms are detailed in Appendix S1.
(b) NTAQ1 phylogenetic analysis. Sequences were retrieved using BLASTP with mouse NTAQ1 as a query. Numbers by phylogenetic branch points give their statistical strength, with 1000 being the highest score. Complete names of indicated organisms are detailed in Appendix S1.

To identify plant proteins that might function as Nt-amidases, we used BLASTP to search public databases for proteins with sequence similarities to *S. cerevisiae* NTA1, mouse NTAN1 and the recently discovered mouse NTAQ1 (Wang *et al.*, 2009). While we did not identify any plant proteins with significant sequence similarities to *S. cerevisiae* NTA1, we detected putative orthologues of mouse NTAN1 in algae [e.g. *Thalassiosira pseudonana* (*E*-value of 2E–04)], as well as in higher plants, in both dicots [e.g. *Arabidopsis thaliana* (*E*-value of 1E-23)] and monocots [for example, *Oryza sativa* (*E*-value of 2E-23)] (Figure 5a). We also found proteins with significant sequence similarities to mouse NTAQ1 in algae [e.g. *T. pseudonana* (*E*-value of 2E-17)], in mosses [e.g. *Physcomitrella patens*, *E*-value of 3E-28)] and in higher plants,

including dicots such as Arabidopsis (*E*-value of 1E-28) and monocots (for example, *O. sativa*; *E*-value of 1E-29) (Figure 5b).

To determine whether these proteins are indeed plant Nt-amidases, we isolated the cDNAs for the Arabidopsis genes *At2g44420* and *At2g41760*, which encode putative Nt^N- and Nt^Q-amidases, respectively, and tested whether their expression could rescue degradation of the N-end rule reporters Asn-βGal and/or Gln-βGal in a *nta1Δ* mutant of *S. cerevisiae* that lacked the endogenous yeast Nt-amidase NTA1 (Baker and Varshavsky, 1995; Kwon *et al.*, 2000). Yeast NTA1 can deamidate both N-terminal Asn and N-terminal Gln; therefore reporters starting with these residues are short-lived in wild-type *S. cerevisiae* and long-lived in its *nta1Δ* mutant. If the At2g44420 protein were indeed an Asn-specific Nt^N-amidase, one would expect Asn-βGal, but not Gln-βGal, to become short-lived in At2g44420-expressing *nta1Δ* yeast. Similarly, if *At2g41760* encoded a Gln-specific Nt^Q-amidase, Gln-βGal, but not Asn-βGal, should become unstable when At2g41760 is expressed in a *nta1Δ* yeast mutant. As expected, expression of yeast NTA1 rescued the *nta1Δ* yeast mutant (Figure 4b). In contrast, expression of the putative Nt^N-amidase At2g44420 led to a reduction in the levels of Asn-βGal, but not of Gln-βGal, suggesting that this protein is specific for substrates with N-terminal Asn (Figure 4b). Expression of the putative Nt^Q-amidase At2g41760 resulted in the opposite response, i.e. degradation of the Gln-βGal reporter, but not of Asn-βGal (Figure 4b). To exclude the possibility that At2g44420 and At2g41760 might catalyze a different modification than the expected deamidation of N-terminal Asn and Gln, we tested whether the degradation of Asn-βGal and Gln-βGal required a functional R-transferase (ATE1), which is required downstream of NTAN1 or NTAQ1 to target these reporters for degradation by the N-end rule pathway (Figure 1). Expression of At2g44420 or At2g41760 together with different Ub-X-βGal reporter constructs in a yeast *ate1Δ nta1Δ* double mutant strain did not result in a reduction of Asn-βGal and Gln-βGal levels (Figure 4c), indicating that ATE1 was indeed required for their degradation in yeast. These results strongly suggest that *At2g44420* and *At2g41760* encode Arabidopsis Nt^N-amidase and Nt^Q-amidase, respectively.

Cys is a tertiary destabilizing residue in plants

Destabilizing activity of N-terminal Cys is mediated by its arginylation. In mammals, and also probably in other eukaryotes that produce NO, the arginylation of N-terminal Cys was shown to require the NO/O_2-mediated oxidation of Cys (Hu *et al.*, 2005; Lee *et al.*, 2005). Chemical considerations and site-directed mutagenesis suggested that the NO/O_2-mediated oxidation step is facilitated by a basic residue at position 2, after N-terminal Cys (Hu *et al.*, 2005). In our Cys–LUC reporter, the second residue is Gln, a non-basic residue. Nevertheless, Cys–LUC is a short-lived protein in tobacco leaves (Figure 2) and in Arabidopsis (Figure 3), suggesting that either a motif for efficient oxidation of N-terminal Cys by NO/O_2 is broader or that in plants, proteins bearing N-terminal Cys are targeted for degradation through a different mechanism (for example, recognition of unoxidized N-terminal Cys by plant R-transferases or by an unknown Cys-specific N-recognin).

To determine whether Cys-bearing N-end rule substrates require functional R-transferases, we compared the relative levels of Cys–LUC reporter protein in *ate1 ate2* double-mutant and wild-type plants that had been stably transformed with a Cys–LUC N-end rule reporter. Western blot analysis of these lines indicated that Cys–LUC accumulated to significant levels in an *ate1 ate2* mutant (Figure 4a), but not in the wild type in which only very low levels of Cys–LUC are detectable (Figures 3 and 4a), indicating that R-transferase activity is required for the destabilizing effect of N-terminal Cys in plants. Although this result strongly suggests that N-terminal Cys requires oxidation prior to its targeting for degradation, it remained possible that, contrary to animal R-transferases, the plant AtATE1 and AtATE2 recognize unoxidized N-terminal Cys. To test this hypothesis, we took advantage of the fact that *S. cerevisiae* lacks NO synthases, which results in Cys being a stabilizing residue in yeast, even in the presence of different isoforms of mouse ATE1 (these isoforms only recognize oxidized Cys in tissues expressing NO-synthases) (Hu *et al.*, 2005, 2006). We expressed *AtATE1* and *AtATE2* in a yeast *ate1Δ* strain and determined whether expression of either R-transferase results in an unstable Cys-βGal reporter. Expression of *AtATE1* or *AtATE2* rescued the *ate1Δ* yeast strain only for the degradation of Glu-βGal and Asp-βGal reporters, whereas Cys-βGal accumulated (Figure 6a), indicating that unoxidized N-terminal Cys is not recognized by the Arabidopsis R-transferases in yeast.

These results suggested that similarly to the mouse isoforms of ATE1, plant R-transferases cannot recognize unoxidized Cys in yeast, and that a motif required for NO/O_2 oxidation of N-terminal Cys does not require a second basic residue. To test whether the destabilizing activity of N-terminal Cys was dependent on the nature of the second residue, we replaced the second residue Gln of the X–LUC reporter with either Lys (a basic residue) or Ser (an uncharged hydrophilic residue) and transiently expressed these reporters in tobacco leaves (Figure 6b). LUC and GUS activities were then measured, and GUS levels were used to correct for transformation efficiency. The relative LUC levels, compared with Met–LUC, showed that Cys is a destabilizing N-terminal residue in tobacco, independently of the nature of the second residue (Figure 6b).

Taken together, our results indicate that Cys is a tertiary destabilizing residue in plants and that N-end rule substrates with N-terminal Cys must be modified (likely through oxidation) before they can be recognized by R-transferases.

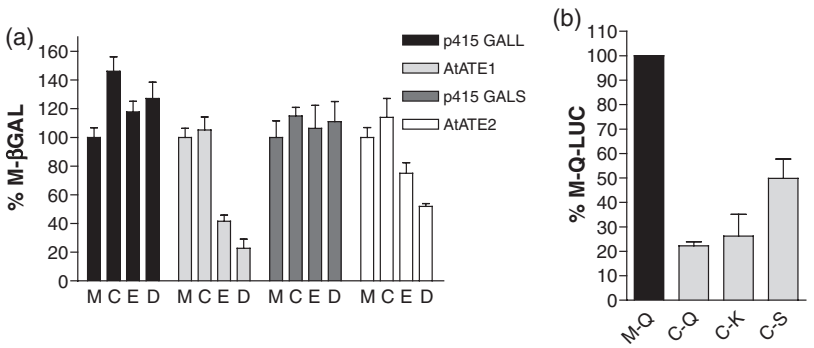

Figure 6. Degradation of Cys-bearing N-end rule reporters.
(a) Expression of *AtATE1* or *AtATE2* is not sufficient to destabilize Cys-βGal in a yeast *ate1Δ*. βGal activities derived from p416 GALL:Ub–X–βGal reporters were measured in the presence of the expression vectors p415 *GALL* HA_6, p415 *GALL:AtATE1-*HA_6, p415 *GALS*, or p415 *GALS:AtATE2-*HA_6. The βGal activities for different N-terminal residues are shown relative to those for Met–βGal. Error bars represent standard errors calculated from three independent experiments.
(b) Influence of the identity of the second residue on the stability of Cys–LUC reporters in tobacco. Tobacco leaves were agroinfiltrated with different Cys–LUC reporter constructs, which varied solely in the nature of their second residue. LUC activities were normalized against the corresponding GUS activities, and thereafter compared with Met–LUC, whose activity was set to 100%. Error bars indicate standard errors of three independent experiments.

DISCUSSION

We used N-end rule reporters, which varied solely in the nature of their N-terminal residue, to determine the full sets of stabilizing and destabilizing N-terminal amino acid residues in plants. While a limited number of such residues had already been described previously, the use of different reporter proteins such as DHFR, GUS and LUC (Bachmair *et al.*, 1993; Potuschak *et al.*, 1998; Worley *et al.*, 1998; Garzon *et al.*, 2007), as well as of different experimental conditions, precluded a direct comparison of the data obtained.

The results of our experiments suggest that the sets of stabilizing and destabilizing residues in plants are similar to those uncovered in mammals. Among the possible differences are Ala, Ser and Thr, which were found to be stabilizing in our agroinfiltration experiments (Figure 2a), but destabilizing in the context of purified X–βGal reporters in reticulocyte extracts (Gonda *et al.*, 1989). However, attempts to identify mammalian E3 Ub ligases that would recognize these N-terminal residues have been unsuccessful, and N-terminal Ala, Ser and Thr are not recognized by the known N-recognins of either the yeast or mammalian N-end rule pathways (Tasaki *et al.*, 2005). In addition, N-terminal Ala, Ser and Thr are often acetylated *in vivo*, a modification that would be expected to preclude their recognition as destabilizing residues (Bradshaw *et al.*, 1998). It is therefore possible that Ala, Ser and Thr are in fact stabilizing residues in mammals (Schnupf *et al.*, 2007).

In summary, we conclude that the sets of stabilizing and destabilizing residues in angiosperms are identical, or almost identical, to those found in mammals. This apparent conservation strongly suggests that the hierarchical organization of the N-end rule pathway might also be conserved between plants and animals. While it had been shown that Asp and Glu are secondary destabilizing residues in plants, the existence of tertiary destabilizing residues had not been demonstrated. We found that X–LUC reporters for Asn, Gln and Cys accumulated to high levels in *ate1 ate2* mutant plants relative to the wild type, suggesting that these residues might indeed be tertiary destabilizing residues in plants. Additional evidence that Asn and Gln are tertiary destabilizing residues was provided by our identification of Arabidopsis orthologues of the mammalian Nt-amidases NTAN1 and NTAQ1. Furthermore, we demonstrated that an N-end rule reporter with N-terminal Cys requires R-transferase activity, but cannot be directly recognized by Arabidopsis R-transferases, suggesting that N-terminal Cys requires a modification (likely oxidation by NO/O_2, as in mammals) before it can be degraded. Taken together, our results indicate that the hierarchical organization of the plant N-end rule pathway is also conserved compared with animals.

Despite the apparent evolutionary conservation of the plant and animal N-end rule pathways, differences exist at the level of the enzymatic components that mediate substrate recognition (N-recognins). Of the two plant N-recognins (PRT1 and PRT6) that have been identified to date, PRT6 has been shown to bind proteins bearing N-terminal Arg, suggesting that it might be specific for the basic (type 1) residues Arg, Lys and His (Garzon *et al.*, 2007). This specificity is in good agreement with the apparent sequence similarities with both yeast and mammalian UBR1, including the presence of the characteristic UBR domain, which has been shown to be necessary and sufficient for the recognition of type 1 residues (Tasaki *et al.*, 2009). The second known plant N-recognin, PRT1, has been shown to recognize hydrophobic N-terminal residues with aromatic side chains (Trp, Tyr and Phe), but not aliphatic hydrophobic residues, such as Leu (and possibly Ile) (Stary *et al.*, 2003). Notably, PRT1 bears no sequence similarities to other N-recognins. BLASTP searches using Arabidopsis PRT1 as a query retrieved putative plant

orthologues from algae such as *Ostreococcus lucimarinus* (*E*-value of 1E-24), moss (e.g. *P. patens*; *E*-value of 4E-62), and monocots (e.g. *O. sativa*; *E*-value of 2E-76), as well as from the protozoa *Toxoplasma gondii* (*E*-value of 1E-31). Thus, PRT1 appears to be present throughout the plant kingdom, as well as in parts of the chromalveolate lineage (e.g. *T. gondii*) (Figure S2), but is not found in animals or fungi.

That the current list of plant N-recognins is incomplete is indicated by the fact that the two above-mentioned N-recognins bind only a subset of all primary destabilizing residues in plants (Figure 1). For example, Leu and Ile have been found to be destabilizing residues (Figure 2), but they are not recognized by either PRT1 or PRT6. Furthermore, it has been suggested that additional N-recognins binding type 1 destabilizing residues remain to be identified (Garzon *et al.*, 2007; Graciet *et al.*, 2009). A strong candidate for a plant N-recognin is the 566-kDa protein BIG (also known as TIR3 or DOC1), which shows sequence similarities to the mouse N-recognin UBR4 (Tasaki *et al.*, 2005).

As described in the Introduction, a variety of functions of the N-end rule pathway and several of its physiological substrates have been discovered in yeast and mammals. In these organisms, the functions of the N-end rule pathway are understood both mechanistically and physiologically ((Tasaki and Kwon, 2007; Varshavsky, 1996), and references therein). The current disposition with plants is midway compared with yeast and animals: while some components of the plant N-end rule pathway have been characterized (Bachmair *et al.*, 1993; Worley *et al.*, 1998; Yoshida *et al.*, 2002; Garzon *et al.*, 2007) and although physiological functions have begun to emerge (Yoshida *et al.*, 2002; Graciet *et al.*, 2009; Holman *et al.*, 2009), substrates of the plant N-end rule pathway remain to be identified. The results of the experiments described above, which yielded the complete sets of stabilizing and destabilizing N-terminal residues, as well as novel enzymatic components of the plant N-end rule pathway, should aid in the systematic identification of substrate proteins.

EXPERIMENTAL PROCEDURES

Plant growth conditions and plant transformation

Plants were grown on a soil:vermiculite:perlite (5:3:2) mixture at 20°C under cool white fluorescent light either at constant illumination or under short-day conditions (8 h light/16 h darkness).

Agrobacterium-mediated plant transformation was carried out using the floral-dip method (Clough and Bent, 1998), and transformants were selected by spraying seedlings with 200 μg ml^{-1} ammonium-glufosinate.

Construction of N-end rule reporter plasmids for plant transformation

A vector (p4204) produced by Worley *et al.* (1998) was used to generate N-end rule reporter constructs with all possible N-terminal residues, as detailed in the supplementary experimental procedures (Appendix S1).

Agroinfiltration of *Nicotiana benthamiana* leaves

Nicotiana benthamiana was grown under continuous light for 4 weeks. *Agrobacterium* transformed with N-end rule reporter constructs were grown for 2 days at 28°C on LB agar supplemented with 100 μg ml^{-1} rifampicin, 100 μg ml^{-1} carbenicillin and 100 μg ml^{-1} spectinomycin. Cells were then resuspended and used for agroinfiltration as described in the supplementary experimental procedures (Appendix S1).

Generation of Arabidopsis stable N-end rule reporter lines

Wild-type Columbia-0 plants and *ate1 ate2* double-mutant plants (Graciet *et al.*, 2009; Holman *et al.*, 2009) were transformed with selected plasmids encoding the Ub–X–LUC reporter and 35S:GUS normalization cassette, which had been used for tobacco agroinfiltration. Different primary transformants (T1 plants) were obtained for each of these reporters and their progeny (T2 generation) was tested for the expression of the GUS normalization reporter, using western blot analysis. Seeds from lines with detectable levels of GUS protein were then propagated and plants homozygous for the X–LUC reporter were selected in the T3 generation, based on the segregation ratios on selective medium (containing the herbicide DL-phosphinothricin). For most residues, several independent lines were tested.

GUS and LUC assays

Measurements of LUC activity were carried out as described in (Luehrsen *et al.*, 1992) using LAR buffer [20 mM tricine, pH7.8, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 0.27 mM coenzyme A (Sigma-Aldrich, http://www.sigmaaldrich.com/), 0.47 mM luciferin (Gold Biotechnology, http://www.goldbio.com), 0.53 mM ATP]. 2 μl protein extract from tobacco leaves (or 1 μl protein extract from wild type or *ate1 ate2* stable N-end rule reporter lines) was added to 100 μl LAR buffer and mixed by pipetting. Luminescence was measured using a FLUOstar OPTIMA instrument (BMG Labtech, http://www.bmglabtech.com) with a delay of 0.5 sec, followed by a 10-sec measurement.

Quantitative GUS activities were measured as described in (Weigel and Glazebrook, 2002) with 4-methylumbelliferyl β-D-glucuronide (MUG) as a substrate, using 2 μl of protein extract from tobacco leaves. For protein extracts made from wild type or *ate1 ate2* stable N-end rule reporter lines, 1 μl of a fivefold dilution was used to measure GUS activity. Fluorescence was measured using a FLUOstar OPTIMA instrument (BMG Labtech), which was calibrated using different concentrations of 4-methylumbelliferone (4-MU; Sigma-Aldrich) ranging from 10 to 300 μM.

Detection of LUC and GUS reporter proteins by western blotting

Protein extracts prepared to test enzymatic activities (see above) were used. Proteins were separated on a 10% SDS–PAGE gel, followed by transfer to a PVDF membrane. LUC was detected using a mouse antibody raised against firefly luciferase (Merck, #OB09, http://www.merck-chemicals.com) diluted 2000-fold in PBS-T [1 × PBS with 0.05% (v/v) Tween 20] supplemented with 5% (w/v) milk. The GUS protein was detected using a rabbit antibody (Molecular Probes, #A5790, http://www.invitrogen.com), which was diluted 1000-fold in 5% (w/v) milk PBS-T.

Construction of yeast N-end rule reporters

The previously published pUB23X N-end rule reporter vectors (Bachmair *et al.*, 1986) were digested with *Xho*I/*Nco*I, followed by

blunting using T4 polymerase. A 3.7-kb fragment encoding the Ub–X–βGal fusion was then ligated into *Sma*I-digested p416 GALL (Mumberg *et al.*, 1994). The resulting plasmids (noted p416 GALL:Ub–X–βGal) were digested to confirm correct orientation of the Ub–X–LUC fusions and then used to test the N-end rule in yeast.

Construction of the *nta1Δ* and *ate1Δ nta1Δ* mutant yeast strain

Yeast strains required for this study were generated as described in the supplementary experimental procedures (Appendix S1).

Cloning of Nt-amidases and R-transferases from *S. cerevisiae* and Arabidopsis

Plasmids used to express Nt-amidases and R-transferases from *S. cerevisiae* and Arabidopsis were constructed as described in the supplementary experimental procedures (Appendix S1).

Yeast experiments

Cells were grown in rich medium (YPD) or in selective medium (SD containing 0.67% yeast nitrogen base without amino acids (Sigma-Aldrich) supplemented with the required auxotrophic nutrients and 3% raffinose). Transformation of *S. cerevisiae* was carried out using the lithium acetate method (Gietz *et al.*, 1992).

To test the N-end rule pathway in yeast, fresh colonies of *nta1Δ*, *ate1Δ nta1Δ* or *ate1Δ* yeast co-transformed with p416 GALL:Ub–X–βGal and p415 derivatives encoding different N-end rule enzymatic components were grown overnight in YNB supplemented with 3% (w/v) raffinose and the required auxotrophic nutrients. This culture was used to inoculate a 3-ml culture of YNB supplemented with 3% (w/v) raffinose and auxotrophic nutrients to an initial OD_{600} approximately 0.1. At OD_{600} approximately 0.6, galactose was added to a final concentration of 1% (w/v) and cells were grown for an additional 4.5 h. After this induction period, 2% (w/v) glucose was added and cells were grown for 30 min. βGal activity measurements were then carried out as described in (Kwon *et al.*, 1999), using red-β-D-galactopyranoside (CPRG; Calbiochem, http://www.merck-chemicals.com) as a substrate.

Phylogenetic analysis

Searches for proteins with sequence similarities to mouse NTAN1, mouse NTAQ1 and Arabidopsis PRT1 were carried out using BLASTP and the NCBI non-redundant protein database. Alignments were produced using CLUSTALX, and distance-based phylogenetic analyses were performed using PHYLIP (Felsenstein, 2005). The PHYLIP program SEQBOOT was used to generate a bootstrapped set of 1000 replicates, which was then submitted to PROTDIST to generate distance matrices, using a Jones–Taylor–Thornton matrix. The resulting distances were then sequentially submitted to NEIGHBOR and CONSENSE, to obtain a consensus tree based on 1000 bootstrap replications of the original alignment. The trees were drawn using TREEVIEW.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Deubiquitylation of Ub–X–LUC reporters and absence of Ub–Pro–LUC accumulation.

Figure S2. Protein distance-based phylogenetic analysis of selected PRT1 homologues.

Table S1. List of plasmids used in this study.

Table S2. List and sequences of oligonucleotides used.

Table S3. Yeast strains used in this study.

Appendix S1. Supplementary experimental procedures.

Data S1. References.

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