Invited Review Article

The plant N-degron pathways of ubiquitin-mediated proteolysis; a review.

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Abstract

The amino-terminal residue of a protein (or amino-terminus of a peptide following protease cleavage) can be an important determinant of its stability, through the Ubiquitin-Proteasome-System associated N-degron pathways. Plants contain a unique combination of N-degron pathways (previously called the N-end rule pathways) E3 ligases, PROTEOLYSIS (PRT)6 and PRT1, recognising non-overlapping sets of amino-terminal residues, and others remain to be identified. Although only very few substrates of PRT1 or PRT6 have been identified, substrates of the oxygen and nitric oxide sensing branch of the PRT6 N-degron pathway include key nuclear-located transcription factors (ETHYLENE RESPONSE FACTOR VIIs and LITTLE ZIPPER 2) and the histone-modifying Polycomb Repressive Complex 2 component VERNALISATION 2. In response to reduced oxygen or nitric oxide levels (and other mechanisms that reduce pathway activity) these stabilised substrates regulate diverse aspects of growth and development, including response to flooding, salinity, vernalisation (cold-induced flowering) and shoot apical meristem function. The N-degron pathways show great promise for use in the improvement of crop performance and for biotechnological applications. Upstream proteases, components of the different pathways and associated substrates still remain to be identified and characterised to fully appreciate how regulation of protein stability through the amino-terminal residue impacts plant biology.

INTRODUCTION, WHAT ARE N-DEGRON PATHWAYS?

N-degron pathways (previously called N-end rule pathways) control the half-life of substrate proteins, determined by the identity and form of the amino-terminal (Nt-) residue (renamed and reviewed in (Varshavsky 2019)). Different types of N-degron pathways are ubiquitous in

living organisms, suggesting that a mechanism for degradation based on identification of the Nt-residue evolved early for regulating protein fate. N-degrons are defined operationally as components of substrate proteins that include an N-terminal 'destabilising' residue, an N-recognin (E3 ligase specific for N-degrons) available N-terminus, and a Lys residue for ubiquitin addition (Gibbs et al. 2016). In eukaryotes N-degron pathways operate in conjunction with the Ubiquitin-Proteasome System (UPS) through pathway-specific E3 ligase N-recognins that shuttle substrates for ubiquitin-mediated degradation. Although physiological functions associated with N-degron pathways remained obscure for a considerable time following their discovery (Bachmair et al. 1986), it is now clear that the pathways exert important influences on cell biology, growth, development and environmental responses in diverse eukaryotes (Turner et al. 2000; Rao et al. 2001; Kwon et al. 2002; Hu et al. 2005; Zenker et al. 2005; Gibbs et al. 2011; Licausi et al. 2011; Cha-Molstad et al. 2017). All genetically-encoded amino-acids at the N-terminus have been shown to have a destabilising influence on substrate stability through N-degron pathways, and in non-plant eukaryotes several branches have been defined including, Arg/, Acetylation (Ac)/, formyl-(f)Met/, Pro/ (Varshavsky 2019) and Gly (Timms et al. 2019) N-degron pathways.

The Arg/N-degron pathway has a hierarchical structure that enzymatically funnels substrates to the N-recognin by sequential alteration of tertiary to secondary destabilising Nt-residues, that are then arginylated to provide an N-terminus with a primary destabilising residue (Figure 1A). Eukaryotic tertiary destabilising residues Gln and Asn can be converted to Glu and Asp by Nt-Glu- (NTAQ1 (Wang et al. 2009)) or Nt-Asn- (NTAN1 (Grigoryev et al. 1996)) amidohydrolases respectively (represented in *Saccharomyces cerevisiae*, hereafter yeast, by a single enzyme NTA1 (Baker and Varshavsky 1995)) and Cys to Cys-sulphinic acid by PLANT CYSTEINE OXIDASE (PCO) enzymes in plants (Weits et al. 2014; White et al. 2017) and an equivalent function in animals, cysteamine (2-aminoethanethiol) dioxygenase (ADO) (Masson et al. 2019). Secondary destabilising residues can be Nt-arginylated by ARGINYL-tRNA TRANSFERASEs (ATEs) to deliver substrates with Nt-Arg- (Manahan and App 1973). In addition to Arg, other amino-acids or modified forms also serve as primary destabilising residues for direct recognition by E3 ligase N-recognins (Figure 1A) (Varshavsky 2019). Physiological substrates with destabilising residues have been identified, but in many cases biochemical components of the pathway and diversity of destabilising residues were initially determined with artificial substrates using the ubiquitin fusion technique (Bachmair et al. 1986) that allows the generation of a stoichiometric mixture of stable and test proteoforms (in this context polypeptides produced after proteolytic cleavage events; (Liu and Moschou 2018)) following constitutive deubiquitinase activity, whose relative stabilities can then be monitored (Bachmair et al. 1986; Graciet et al. 2009). Analysis of the half-lives of artificial N-degron pathway substrates in plants (*Arabidopsis thaliana*, hereafter arabidopsis and

Nicotiana tabacum, tobacco) showed that destabilising residue identity is conserved with higher animals, including the observation of Nt-Cys as a destabilising residue (Graciet et al. 2010). This indicated that enzyme systems leading to degradation of substrates are conserved.

ENZYMATIC COMPONENTS OF PLANT N-DEGRON PATHWAYS

Known and unknown plant E3 ligase N-recognins mediate substrate degradation

A major difference between plant and non-plant eukaryotic N-degron pathways is the involvement of different classes of E3 ligase N-recognins that account for the recognition of the major classes of destabilising residues. In non-plant systems single protein E3 ligases recognise both type 1 (basic, including Arg) and type 2 (hydrophobic bulky) residues of the Arg/N-degron pathway, with yeast UBR1 and mammalian cognate UBR-type ligases containing recognition domains for type 1 (UBR box) and type 2 (ClpS-like domain) residues (Tasaki et al. 2012) (Figure 1B). However, a screen in arabidopsis to identify genetic components responsible for degradation of substrates with Nt-Phe identified PROTEOLYSIS (PRT)1, a protein with a unique combination of structural domains, comprising two RING finger and one ZZ domain (Bachmair et al. 1993; Potuschak et al. 1998) (Figure 1B). Further analysis showed that PRT1 acts as a ubiquitin ligase and only recognises substrates with Nt-aromatic (Phe-, Tyr- and Trp-) residues (Stary et al. 2003; Mot et al. 2018). This indicated that the ClpS-like domain function of UBR1 has been replaced in plants with a novel domain-combination acting within a separate ligase. Arabidopsis contains at least three proteins with UBR-like domains. One of these, PRT6, with greatest similarity to UBR1 was shown to mediate degradation of Nt-Arg, but not of Phe- or Leu-, and PRT6 does not contain a ClpS-like domain (Garzon et al. 2007) (Figure 1B). The N-degron pathway-related functions of the other two, BIG and AT4G23860 (Figure 1B), have not been reported. Although the existence of a plant Ac/N-degron pathway has not yet been shown, proteins with sequence similarities to yeast E3 ligase Ac/N-recognins have been identified (Figure 1C), including ER-associated Doa10 and cytoplasmic Not4 (Hwang et al. 2010; Shemorry et al. 2013). As yet no Pro/N-degron pathway has been shown in plants, though Nt-Pro was shown to be destabilising as a Pro-LUCIFERASE fusion in tobacco (Graciet et al. 2010), and the stability of Nt-Met- and Nt-formyl-Met and Gly-N-degron containing proteoforms have not yet been determined in plants.

Observation of multiple N-recognins indicate that in plants N-degron pathways and substrates degraded by them are regulated in a more complex manner than other eukaryotes. It indicates the separation of biological roles for degradation of different cohorts of substrates with different Nt-destabilising residues. The observation that neither PRT6 nor PRT1 regulate

the degradation of substrates with Nt-Leu (Garzon et al. 2007) suggests that recognition of Nt-Leu requires additional yet-to-be discovered N-recognin components. The terminology for N-degron pathways used in mammalian/yeast systems is not suitable in plants because of the separation of functions of N-recognins into more than 2 distinct proteins. Therefore, it is proposed the plant N-degron pathways are named by the associated N-recognin (e.g. PRT6 or PRT1 N-degron pathway) or by the destabilising residue where there is no known N-recognin (e.g. Leu/N-degron pathway), and branches of the pathways by associated enzymes (e.g. PCO/NTAQ1/NTAN1/ATE branches of the PRT6 N-degron pathway) (Millar et al. 2019).

Discriminatory enzyme pathways funnel destabilising residue-specific substrates to PRT6

Tertiary and secondary destabilising residues are converted to primary destabilising residues through the action of Nt-residue-specific enzyme pathways (Figure 1A). Conversion of the N-terminus from secondary to the primary destabilising residue Arg in arabidopsis is controlled by ATE activity (Yoshida et al. 2002). ATE1 was shown in protoplasts from mesophyll cells to enhance degradation of artificial substrates with Nt-Asp and -Glu (Yoshida et al. 2002). Interestingly, although arabidopsis contains two ATE genes, in the accession Wassilewskaja (Ws) ATE2 contains an insertion of a single base resulting in a premature stop codon (Riber et al. 2015). In accession Columbia (Col)-0 differential effects of the two loci were observed in regulating germination (Holman et al. 2009), and ATE1 was shown to be responsible for the majority of total ATE activity (Graciet et al. 2009), indicating evolutionary instability of ATE2 function. In addition, in other taxonomically diverse model species such as rice (flowering plant monocot) and *Physcomitrella patens* (hereafter moss, a bryophyte), ATE is represented as a single gene (Goff et al. 2002; Schuessele et al. 2016). Both Nt-Asn and -Gln were shown to be destabilising in *Agrobacterium tumifaciens*-infiltrated tobacco leaves and in an ATE-dependent way in stably-transformed arabidopsis plants (Graciet et al. 2010). Sequence analysis indicated that the two arabidopsis genes At2g44420 and At2g41760 encode putative Nt-Asn (NTAN1) and Nt-Gln (NTAQ1)-amidohydrolases respectively, and these were shown to direct the degradation of cognate Nt-residues in an ATE-dependent manner in the yeast *nta1* mutant (that lacks both amidase activities) (Graciet et al. 2010). Subsequently, in vitro analysis of the Nt-deamidation capacity of recombinant arabidopsis NTAQ1 demonstrated a high specificity for Nt-Gln compared to Nt-Asn, Gly- and Lys- (Vicente et al. 2019). The formation of Nt-pyroglutamate by glutaminyl cyclase (GC) was suggested as a possible competition for Nt-deamidation by NTAQ1 (Wang et al. 2009), however, in arabidopsis analysis of response to the pathogen *Pseudomonas syringae* pv *tomato* DC3000 (*Pst*) of *ntaq1* and a mutant of *GLUTAMINYL CYCLASE1* mutant (*gc1*) indicated that for this response such a competition is unlikely (Vicente et al. 2019).

Plant cysteine oxidases (PCOs) as oxygen sensors

Although Nt-Cys is a tertiary destabilising residue in mammalian systems, it was not initially clear if conversion to a secondary destabilising residue was enzymically controlled or not (Hu et al. 2005; Lee et al. 2005). Nt-Cys as part of an N-degron can be destabilising in plants, and as in mammals this is dependent on the presence of both oxygen and nitric oxide in vivo (Gibbs et al. 2014b). It was considered that oxidation of cysteine to Cys-sulphinic ($-SO_2^-$) or -sulphonic ($-SO_3^-$) acid provided a bioisostere of Asp thereby acting as a substrate for ATE (Kwon et al. 2002). A breakthrough in the field was the discovery that in plants oxidation of Nt-Cys requires the activity of PCO enzymes (Weits et al. 2014). These are monomeric, non-heme iron-dependent dioxygenases that use molecular oxygen to catalyse the formation of Cys-sulphinic acid from Nt-Cys, that has been shown to then provide an efficient substrate for arabidopsis ATE1 in vitro (White et al. 2017). The arabidopsis genome encodes five *PCO* genes and each protein has been shown to have discrete preferences for the five Nt-Cys ERFVII transcription factor and VRN2 substrates (Gibbs et al. 2018; White et al. 2018). Importantly, the PCOs have K_m^{app} (O_2) values that are within a physiologically relevant range for response to external or internal oxygen deficit, indicating that they have the capacity to function as oxygen sensors for ecologically important variables, including flooding (White et al. 2018). Following the identification of plant enzymes, an animal ADO enzyme with similar function in regulating mammalian Nt-Cys substrates was identified (Masson et al. 2019).

How is NO required in vivo for degradation through the PCO branch of the PRT6 N-degron pathway?

In both mammalian and plant systems NO, in addition to oxygen, was shown to be absolutely required for degradation of Nt-Cys substrates in vivo (Hu et al. 2005; Gibbs et al. 2014b) and in a reconstituted in-vitro system (Hu et al. 2005) (Figure 1A). However, in vitro assessment of the activity of PCOs (Weits et al. 2014; White et al. 2017; White et al. 2018), and analysis of a reconstituted plant PCO-regulated system for Nt-Cys degradation in yeast (Puerta et al. 2019) demonstrated that oxidation leading to degradation is possible in the absence of NO (*Saccharomyces cerevisiae* has not been shown to produce endogenous NO under standard growth conditions). It therefore remains to be discovered how NO exerts a positive influence on degradation of Nt-Cys substrates in a cellular context, that may include an effect on the activities of enzymic components of the pathway (for example it was recently shown that PRT6 contains a putative NO-binding domain (Zarban et al. 2019)) or indirectly through alterations of cellular energy balance (Greenway and Armstrong 2018).

Organellar N-degron pathways in plants

Both chloroplasts and mitochondria are bacterially-derived, and bacteria contain well-defined N-degron pathways (Tobias et al. 1991) regulated by the caseinolytic protease (Clp)AP AAA+ chaperone-protease system, that requires the ClpS adaptor N-recognin for recognition of and binding to destabilising Nt-residues. Non-plant (UBR-type) eukaryotic N-recognins contain a ClpS-like region (Lupas and Koretke 2003). Both primary and secondary destabilising residues exist in bacterial systems, and there may be diversity in secondary destabilising residue types, converted to primary destabilising residues through distinct transferase activities (Graciet et al. 2006). Both mitochondria and chloroplasts contain Clp-like components (Peltier et al. 2004), including the presence of a chloroplast ClpS-like protein (ClpS1), and the chloroplast Clp system is necessary for normal growth and development (Nishimura and van Wijk 2015). Amino-terminal proteomic analysis of the chloroplast stroma indicated that peptides with specific residues at the N-terminus were underrepresented (Rowland et al. 2015), and reporter proteins in transplastomic plants showed differential stabilities dependant on their Nt-residues (Apel et al. 2010), suggesting that N-degron pathway(s) operate in the chloroplast. Recently ClpS1 specificity was investigated in vitro and recognition assays for GFP-fusion proteins bearing defined Nt-residues indicated a low specificity for bacterial N-degrons, but high specificity recognition for Nt-Phe or Nt-Trp (Colombo et al. 2018; Montandon et al. 2019). These studies suggest a chloroplast N-degron pathway with high specificity, though no substrates or transferase activities have yet been identified.

PROTEIN SUBSTRATES OF THE PLANT N-DEGRON PATHWAYS

Although the enzymatic components of N-degron pathways are highly conserved in higher eukaryotes, physiological substrates of the pathways are diverse. Substrates with destabilising residues of all branches have been identified in yeast and mammalian systems (Varshavsky 2019), but this is not the case in plants, where to date very few substrates have been defined (Table 1). In general definition of substrates is problematic starting from only knowledge of pathway residue-specificities, and the total number of known substrates in all systems is not large. However, new proteomics methodologies that allow the identification of Nt-residues are likely to provide an important tool for increasing identification of substrates (Perrar et al. 2019).

Proteases and their role as checkpoints initiating N-degron pathways

A key pre-requisite for entry into the majority of N-degron pathways (but not necessarily Met- or formyl-Met pathways, Figure 1A) is the action of intracellular peptidases to cleave their targets, producing proteoforms with exposed N-degrons. In mammalian systems caspase and calpain endo-protease derived proteoforms have been shown to be degraded through the

Arg/N-degron pathway and are associated with important cellular processes (Ditzel et al. 2003; Piatkov et al. 2012; Brower et al. 2013; Chui et al. 2019; Sandstrom et al. 2019). In addition, the exo-peptidase MetAP (Figure 1A) acts a checkpoint for entry into N-degron pathways when the second residue of a nascent protein is a destabilising residue. The arabidopsis genome contains over 700 proteases, though only a handful of targets have been described (Tsiatsiani et al. 2012) suggesting that many proteoform substrates of N-degron pathways remain to be discovered.

Methionine amino-peptidase, the 'MC-ome' and oxygen-sensing substrates: The initiating Met-1 of nascent proteins is irreversibly cleaved in Nt-Met excision (NME), revealing the second residue as the new amino-terminus of the protein, and this reaction can be carried out by MetAP. The classically described substrate specificity of MetAP includes a preference for small side chain second residues (including Ala, Ser, Gly, Thr, Cys, Pro, or Val), of which Cys-, Gly and Pro- have been described as destabilising residues. Recent data indicate that these are only destabilising in specific sequence contexts, i.e. not all proteins with these residues at position 2 will be destabilised via N-degron pathways (Kats et al. 2018; Timms et al. 2019). The arabidopsis and rice genomes contain 246 and 421 gene models, respectively, that initiate Met-Cys and have the capacity to act as substrates of the PCO branch of the PRT6 N-degron pathway (Figure 1A). Interestingly, like Gly (Timms et al. 2019) Cys at position 2 appears evolutionarily restricted in diverse eukaryotic taxonomic groups, perhaps because for Cys this provides the capacity for these proteins to be N-degron pathway substrates as part of oxygen/NO sensing systems (Gibbs et al. 2014a). These proteins (collectively the 'MC-ome') represent diverse functions, many species specific, but a large number also conserved across flowering plants. Amongst these are three Met-Cys initiating transcription-associated protein types that have been shown to be bona fide substrates of the PCO branch of the PRT6 N-degron pathway, the Group VII ETHYLENE RESPONSE FACTOR (ERFVII) transcription factors (Gibbs et al. 2011; Licausi et al. 2011), the Polycomb Repressive Complex 2 component VERNALISATION (VRN)2 (Gibbs et al. 2018) and LITTLE ZIPPER 2 (ZPR2) transcription factor (Weits et al. 2019). In all three cases it was shown physiologically that stability of the proteins is enhanced in hypoxia (reduced atmospheric oxygen) and genetically that this is mediated by the PRT6 N-degron pathway. As predicted by observations in mammalian systems (Hu et al. 2005; Lee et al. 2005) it was also shown that in vivo NO, in addition to atmospheric oxygen, is also required for destabilisation of ERFVIIs and VRN2 through this pathway (Gibbs et al. 2014b; Gibbs et al. 2018). Despite these impressive advances in understanding how stability of Cys N-degron substrates control plant growth and development, direct proof of in vivo chemical modifications to Nt-Cys of known substrates in response to oxygen have yet to be reported. Many other MC-ome proteins remain to be

investigated, though due to the strict structural requirements for N-degron function, it is likely that only a small proportion of these will be physiological PRT6 N-degron pathway substrates. For example, it was shown that the arabidopsis Met-Cys- initiating proteins MADS AFFECTING FLOWERING(MAF)5 (Gibbs et al. 2011), and ERF#111 (Bäumler et al. 2019) are not N-degron pathway substrates. For VRN2 evolutionary entry into the PRT6 N-degron pathway occurred specifically in flowering plants following duplication of the pre-existing *EMBRYONIC FLOWER* (*EMF*)*2* gene that resulted in deletion of the Nt-cap domain and exposure of a pre-existing internal Met-Cys at the new N-terminus (Gibbs et al. 2018). Recent work has indicated that in yeast NME may also expose other destabilising residues (Nguyen et al. 2019), suggesting that destabilising residues in addition to Cys at position 2 may also be naturally exposed. Interestingly in *Physcomitrella patens* Acylamino-acid-releasing enzyme (PpAARE) was shown to be a target of ATE through Asp-2 and perhaps a PRT6 N-degron pathway substrate (Hoernstein et al. 2016).

Endopeptidase derived substrates of N-degron pathways: In plants only a single example exists of an N-degron pathway substrate derived from endopeptidase action. Two E3 ligases, BIG BROTHER (BB) and DA2, regulate cell proliferation during organ growth and were shown to interact genetically with DA1 (Xia et al. 2013). Subsequently it was shown that DA1 is an endopeptidase that is activated via BB and DA2 ubiquitylation, and that activated DA1 cleaves a proportion of the BB/DA2 protein population (Dong et al. 2017). In the case of BB, Edman degradation suggested that cleavage revealed Nt-Tyr61. Analysis in vitro using ubiquitin fusion constructs in rabbit reticulocyte lysate, and in protoplasts comparing wild-type and *prt1* mutants, indicated a short half-life for the carboxyl-terminus (Ct-) proteoform of BB that was dependant on Tyr61 and *PRT1*. In addition, PRT1 was shown in vitro to bind to a peptide mimicking Try61-BB. These data indicate that the PRT1 N-degron pathway is one mechanism controlling the stability of BB Ct-proteoform produced following DA1-mediated cleavage. As DA1 cleaves other proteins associated with cell proliferation, PRT1 may also be involved in controlling their specificity, and it will be interesting to determine whether there is a role for PRT1 in cell proliferation.

PHYSIOLOGICAL ROLES OF N-DEGRON PATHWAYS IN PLANT GROWTH AND DEVELOPMENT AND ENVIRONMENTAL INTERACTIONS

Although the first identified component of plant N-degron pathways (PRT1) was cloned more than 20 years ago (Potuschak et al. 1998), the importance of physiological roles for the N-degron pathways in growth and development has only become apparent in the last decade (Table 1). In particular it has become clear that the pathways play key roles in plant-environment interactions by controlling the half-lives of known regulator proteins, or through

yet to be identified substrates. Physiological roles were first identified by analysis of phenotypes of mutants obtained through either forward or reverse genetic screens. The first genetically-identified component of plant N-degron pathways shown to regulate a physiological process was ATE1, as a determinant of leaf senescence (from the mutant *delayed-leaf-senescence* (*dls*)1) (Yoshida et al. 2002). *DLS1* (that encodes ATE1) was shown to influence the progression of leaf senescence in an age-dependant way and in response to dark, indicating that substrate(s) that require Nt-Arg addition are required for this process. Subsequently it was also shown that a double mutant *ate1 ate2*, that removes all ATE activity, influenced the development of rosette leaves, phyllotaxis and internode elongation, suggesting that cell elongation is impaired in the mutant (Graciet et al. 2009). *ATE* genetic activity was shown to act redundantly with the *ASYMMETRIC LEAVES1* (*AS1*) transcription factor, but independently of auxin, to control these aspects of leaf development. MetAP, PRT6 and ATE1/2 were shown to be key regulators of seed germination, controlling dry seed after-ripening, seedling sugar sensitivity, abscisic acid (ABA) sensitivity of germination and seedling lipid breakdown during establishment (Holman et al. 2009; Gibbs et al. 2014b). The moss ATE gene was also shown to contribute to gametophytic development and be responsive to ABA (Schuessele et al. 2016). Genetic analysis indicated that PRT6 N-degron pathway function inhibits the activities of three ABA-associated transcription factors that promote dormancy, ABA INSENSITIVE (ABI)3, ABI4 and ABI5, but that N-degron pathway regulation of oil bodies was not ABA dependent (Holman et al. 2009; Zhang et al. 2018b). These data indicate that the PRT6 N-degron pathway inactivates core component(s) that inhibit germination. Ethylene-stimulated germination was shown to be abolished in *prt6* and *ate1 ate2* mutants indicating an interaction between this hormone and the PRT6 pathway (Wang et al. 2018). The PRT6 N-degron pathway was also shown to influence the proteome of etiolated (dark germinated) seedlings, with up-regulation of 45 protein groups in *prt6*, including ERFVII-regulated storage proteins (Zhang et al. 2018a).

Physiological roles of the PCO branch of the PRT6 N-degron pathway:

Work in mammals showed that UBR-mediated oxygen and NO sensing via Met-Cys initiating REGULATOR OF G PROTEIN SIGNALLING (RGS)4,5 and 16 proteins was physiologically important (Kwon et al. 2002; Hu et al. 2005; Lee et al. 2005; Lee et al. 2012). Analysis of the analogous pathway in plants has also shown key physiological functions. In most cases these are clearly related to changes in the physiological availability of oxygen or NO (as both are required in vivo to destabilise substrates) but in other cases there is not an obvious link. For example, stabilisation of VRN2 occurs when seedlings are grown at low temperature (below 5C) and VRN2 has an essential role in the control of cold-induced flowering, indicating that this stabilisation is an important functional requirement (Gibbs et al. 2018). It is possible that

cold induces an energy crisis (Greenway and Armstrong 2018), as enzymes associated with low-oxygen responses are also induced in the cold, suggesting mitochondrial involvement, or another possibility could be that lower temperature reduces enzyme activities of the pathway, particularly PCOs (Gibbs et al. 2018). ERFVIIs were shown to strongly influence flowering time in a *PRT6*-dependant manner (Vicente et al. 2017), that might be related to changes in NO levels, but a previous publication suggests an opposite relationship (He et al. 2004). In poplar (*Populus* spp.), Zn^{2+} excess in the root environment was shown to induce a hypoxia-like molecular response (Dalle Carbonare et al. 2019), suggesting other mechanisms controlling stability. High zinc levels resulted in the stabilisation of the poplar ERFVII Pop_ERFB2-1 in transiently-transfected poplar mesophyll protoplasts, and in vitro zinc was shown to inhibit PCO enzyme activity, suggesting that in soils with high zinc levels physiological responses may be determined by inhibition of the PCO branch of the PRT6 N-degron pathway and subsequent stabilisation of associated substrates. Clearly more understanding is needed of the influence of external factors on enzymes of the PCO branch of the PRT6 pathway to understand how plants use the pathway to sense environmental change. In addition an age-dependence of ERFVII and PRT6 activity was shown for the induction of hypoxia genes in arabidopsis, indicating that internal developmental signals may also influence pathway and/or substrate functions (Giuntoli et al. 2017; Vicente et al. 2019).

Oxygen as an external signal: Two reports identified the PRT6 N-degron pathway, and ERFVII transcription factors, as key regulators of plant oxygen sensing (Gibbs et al. 2011; Licausi et al. 2011). The five arabidopsis ERFVIIs (RELATED TO AP (RAP)2.12, 2.2, 2.3, HYPOXIA RESPONSIVE ERF (HRE)1 AND HRE2) were shown to act as efficient substrates for the PRT6 N-degron pathway. The ERFVIIs have been shown to regulate responses to many abiotic and biotic inputs (Gibbs et al. 2015), but these two publications demonstrated the importance of the PRT6 N-degron pathway in their regulation by oxygen as an external signal. In arabidopsis, the transcriptomes of *prt6* and *ate1 ate2* mutant seedlings were shown to resemble that of seedlings under hypoxia, with many core hypoxia genes (Mustroph et al. 2010) constitutively up-regulated, resulting in enhanced survival of hypoxia (Gibbs et al. 2011). Mutant barley (*Hordeum vulgare*) plants with reduced PRT6 function also showed enhanced tolerance to waterlogging (Figure 2A) and constitutively expressed hypoxia genes (Mendiondo et al. 2016). In arabidopsis, stability of ERFVIIs was enhanced in *prt6* and *ate1 ate2*, and by hypoxia (Kosmacz et al. 2015), and this was shown to be dependent on Cys2 (Gibbs et al. 2011). The ERFVII proteins are highly conserved in flowering plants (Nakano et al. 2006), and rice ERFVIIs include SUBMERGENCE(SUB)1A and SNORKEL (SKL)1 and SKL2, that have been shown to enhance tolerance to rapid or deep-water flooding respectively (Xu et al. 2006; Hattori et al. 2009). Interestingly although SUB1A protein contains Cys2 it was shown that it

is not a substrate for the PRT6 N-degron pathway in vitro (Gibbs et al. 2011), because the C-terminus protects the N-terminus by directly interacting (Lin et al. 2019), providing a possible explanation for the improved flood-tolerance of accessions containing this locus. Two other rice ERFVIIs, 66 and 67 were shown to act downstream of SUB1A, and to be targets of N-degron pathway-mediated degradation (Lin et al. 2019). In addition to ERFVIIs, the Cys2 substrate VRN2 was also shown to enhance tolerance to seedling hypoxia and plant waterlogging (Gibbs et al. 2018), indicating that diverse members of the 'MC-ome' may contribute to increased tolerance of flowering plants to low oxygen (Holdsworth 2017). External oxygen levels decline as a consequence of flooding, and also decline below the soil level. Following germination, in the absence of light under the soil-surface, flowering plants go through a skotomorphogenic developmental programme, characterised by hypocotyl elongation, apical hook formation and inhibition of chlorophyll accumulation in cotyledons (Abbas et al. 2013). Oxygen sensing through the PCO branch of the PRT6 N-degron pathway was shown to be a key regulator of this response, mediated by ERFVIIs (Gibbs et al. 2014b; Abbas et al. 2015) (Figure 2B,C). Hypoxia inhibited apical-hook opening and chlorophyll accumulation in an ERFVII-dependant manner, and the response was shown to be conserved across dicot lineages. Sensing oxygen during skotomorphogenesis was shown to be essential, as low oxygen during dark establishment greatly enhanced survival following subsequent transfer to the light (Abbas et al. 2015) (Figure 2B). Riber *et al.* also observed that an allele of *prt6*, *greening after extended darkness1* (*ged1*) showed survival of extended dark, and that *prt6* and *ate1 ate2* mutants were more tolerant of starvation conditions (Riber et al. 2015).

Oxygen as an internal signal: In addition to the importance of correct responses to changes in external oxygen levels, a large body of work demonstrates that oxygen levels within plant tissues vary greatly that has regulatory consequences, for example in germ-cell fate (Kelliher and Walbot 2012), suggesting that this may be an important determinant controlling metabolism and development. Recently a micro-scale oxygen electrode was used to demonstrate that a steep oxygen gradient exists around the shoot apical meristem (SAM) of arabidopsis and the distantly-related asterid dicot *Solanum lycopersicum*, leading to very low meristem oxygen levels, that was not observed in the root meristem (Weits et al. 2019). This was correlated with increased expression of core hypoxia genes within the SAM. Exposure of plants to hyperoxia (above sea level atmospheric levels of oxygen) inhibited organ formation, demonstrating (as for skotomorphogenic development (Abbas et al. 2015)) that hypoxia is an important determinant for correct developmental pathways. The ZPR2 Cys2 substrate was shown to be expressed and stable specifically in the SAM (Figure 2D), to repress the expression of a group of class-III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP III)

transcription factors involved in regulating SAM activity, and to thereby genetically control leaf initiation rate. Stabilisation of VRN2 was also restricted to meristem regions in a PRT6 N-degron pathway specific manner (Gibbs et al. 2018) though differently to ZPR2, whose promoter only drives expression in the SAM, VRN2 stabilisation requires PRT6 activity to restrict it to the apical meristem regions of shoots and roots (Figure 2D). In addition, stabilised ERFVIIs in hypoxic niches, that are established during lateral root development, result in the repression of auxin-induced genes and regulation of lateral root production (Shukla et al. 2019), and RAP2.12, like VRN2, is restricted to meristem regions of root tissues (Figure 2D). Internal oxygen sensing through ERFVIIs has also been shown to be an important component of plant pathogen interactions (see below).

These data demonstrate an essential role for oxygen sensing through the PRT6 N-degron pathway during development, the importance of hypoxic niches for correct development, and suggest that other "MC-ome" substrates (or other substrates with Nt-Cys) remain to be discovered with roles in hypoxic niches (Holdsworth 2017).

NO sensing and signalling mediated by Nt-Cys N-degrons

Both oxygen and NO are required in vivo for degradation of Nt-Cys substrates in mammalian systems (Hu et al. 2005; Lee et al. 2005). This led to the investigation and discovery of the importance of NO for degradation of ERFVIIs, and of the PRT6 N-degron pathway in NO sensing (Gibbs et al. 2014b). It was shown that stability of ERFVIIs RAP2.3 and HRE2 and an artificial Cys2 substrate (C-^{HA}GUS) were regulated by NO availability through Cys2 (Gibbs et al. 2014b; Vicente et al. 2017). NO was previously shown to enhance seed germination by breaking dormancy of imbibed seeds (Bethke et al. 2007), and both *prt6* and *ate1 ate2* mutants were shown to be resistant to NO-dormancy breaking in an ERFVII-dependant manner (Gibbs et al. 2014b). ERFVIIs were shown to bind the 5' UTR region of the *ABI5* gene in the covering endosperm layer of the seed and thereby enhance seed dormancy and ABA sensitivity of germination. Further analyses showed that ERFVIIs regulate diverse NO responses in the PRT6-dependant way throughout growth and development, including stomatal aperture, hypocotyl elongation (Figure 2C) (Gibbs et al. 2014b) and apical hook opening during skotomorphogenesis (Abbas et al. 2015). As would be expected VRN2 stability was also shown to be regulated by NO, but no physiological importance has been associated with this (Gibbs et al. 2018). Higher plants do not contain an NO synthase, and NO synthesis mainly occurs through a side reaction of NITRATE REDUCTASE (NR) to reduce nitrite. Abiotic stresses (including exposure to drought and salinity) reduce NR activity, and this was shown in arabidopsis and barley to enhance stability of an artificial reporter of the PCO branch of the PRT6 N-degron pathway (Gibbs et al. 2014b), and ERFVIIs enhanced tolerance to these

stresses in a PRT6 N-degron pathway regulated manner in arabidopsis and in a *PRT6* dependant way in barley (Figure 2E) (Vicente et al. 2017).

A coupling between the three gases ethylene, NO and oxygen was recently shown to rapidly control plant response to low oxygen (Hartman et al. 2019). In normoxia, accumulation of the gaseous phytohormone ethylene (that occurs as a passive response to flooding) was shown to enhance expression of the NO-scavenging protein PHYTOGLOBIN (PGB)1, leading to removal of cellular NO and consequent stabilisation of ERFVIIs including RAP2.12 (Figure 2F). Interestingly this occurred without a large induction of ERFVII-regulated hypoxia-enhanced transcripts, that occurred in an ERFVII-dependent way only after subsequent hypoxia, and to a much greater extent than without ethylene pre-treatment, indicating pre-adaptation to flooding via ethylene sensing and downstream response (Hartman et al. 2019). Thus, plants use both oxygen and NO gases (in combination for some responses also with ethylene) to sense and respond to a range of abiotic stresses associated with water availability.

Roles for N-degron pathways in plant-pathogen interactions

Both the PRT1 and PRT6 N-degron pathways have been shown to influence plant-pathogen interactions though different reports have reached opposite conclusions about the roles of the pathways in controlling the plant immune-response to the necrotroph *Botrytis cinerea* and the hemi-biotroph bacterium *Pst*. Initially it was reported that alleles of *prt6*, *ate1 ate2*, *ntaq1* and *prt1* were equally or more sensitive than wild-type plants to a range of pathogens with different lifestyles plants (de Marchi et al. 2016). More recently it was shown that removal of PRT6 pathway function enhanced tolerance to *Pst* in arabidopsis, and to *Pseudomonas syringae* pv *japonica* in barley with reduced *PRT6* expression (Vicente et al. 2019). In arabidopsis this was shown to occur through *NTAQ1*, providing a first molecular physiological function for this branch in plants, through enhanced expression in untreated plants of defence genes and increased levels of synthesis proteins for the phytoalexin camalexin. ERFVIIs were also shown to regulate stomatal closure in response to *Pst*, as the *erfVII* mutant (a pentuple mutant lacking ERFVII activity, *rap2.12 rap2.2 rap2.3 hre1 hre2*, abbreviated to *erfVII* (Marin-de la Rosa et al. 2014; Abbas et al. 2015)) failed to close stomata upon recognition of the pathogen (Vicente et al. 2019). ERFVIIs may be stabilised following infection in response to a pathogen-induced sharp decline in NR activity, that was shown to stabilise an artificial Nt-Cys reporter in both arabidopsis and barley (Vicente et al. 2019).

A link was observed between the PCO branch of the PRT6 N-degron pathway/ERFVII oxygen-sensing system and plant pathogen interactions in two publications, indicating a role for pathogen manipulation of the hypoxia sensing and response system as a mechanism for attack. Fermentation-associated genes are often upregulated in plants in response to gall-

forming pathogens, and gall formation by and susceptibility to the protist *Plasmodiophora brassicae* was increased in *prt6* and *ate1 ate2* mutants, where ERFVIIs are constitutively stabilized (Gravot et al. 2016). However, in the absence of ERFVII function (in the *prt6 erfVII* mutant) gall development was greatly reduced compared to wild-type (Figure 2G). An assessment of publicly available datasets demonstrated that hypoxia-induced transcriptomes are a common signature in tumour-forming plant pathogens, including *Agrobacterium tumefaciens*. The high metabolic demand of *A. tumefaciens* crown-gall formation was shown to produce a steep hypoxia gradient within the gall, leading to up-regulation of hypoxia-responsive genes (Kerpen et al. 2019). Crown gall symptoms were greatly reduced in the *erfVII* mutant but increased in mutants with constitutively stabilised ERFVIIs (*pco1 pco2*, *ate1 ate2* and *prt6*). These data Indicate that tumour-forming pathogens utilise the endogenous plant oxygen sensing mechanism to enhance infection success, and also raise the interesting possibility that other Nt-Cys substrates may also be stabilised in response to pathogen attack and influence the host response.

Proteoforms derived from the arabidopsis protein RPM1-INTERACTING PROTEIN (RIN)4 were previously hypothesised to be substrates of NTAN1 and ATE branches of the PRT6 N-degron pathway (Takemoto and Jones 2005). This is because following cleavage by *Pst* type III effector protein cysteine protease AvrRpt2 two cleavage products with Nt-Asn or Nt-Asp are revealed. A recent analysis suggested that these are not physiological substrates of these pathways, based on the lack of accumulation of these cleavage products in *prt6* or *ate1 ate2* protoplasts (Goslin et al. 2019), though alternative internal degrons might also degrade these fragments. Based on the cleavage sites of AvrRpt2 several arabidopsis nitrate-induced (NOI) domain family proteins were identified as possible substrates, that were predicted to reveal secondary destabilising residues Asp and Glu (Chisholm et al. 2005). Analysis of these 10 predicted cleavage sites in arabidopsis protoplasts indicated that cleavage of NOI1, 6 and 11 may generate substrates of the ATE branch of the PRT6 pathway (Goslin et al. 2019), and it will be important to understand the physiological relevance of this regulation in plant defence.

Several recent reports indicate the presence of an Ac/N-degron pathway in plants, that may be associated with plant-pathogen interactions. Plant proteins with sequence-homology to yeast Ac/N-degron N-recognins exist (Figure 1C) and have been shown to regulate plant growth and development (Zhao et al. 2014). Nt-acetylation was shown to be key mechanism controlling the stability of an arabidopsis Nod-like immune-receptor (NLR) SUPPRESSOR OF NPR1, CONSTITUTIVE 1 (SNC1) (Xu et al. 2015). The SNC1 gene produces distinct Nt-isoform proteins derived from both alternative translational-initiation of the Met1-Met2 initiating protein, and N-terminal acetylation. Acetylation by different N-terminal acetyltransferase (NAT) complexes led to different fates. Acetylation of Met1 via NatA enhanced degradation,

whereas Nt-Met2 acetylation by NatB stabilised SNC1, demonstrating antagonistic effects of Nt-acetylation on protein fate, and indicating that an Ac/N-degron pathway with important physiological functions may exist in plants. This regulation has an effect in the plant immune response, as mutation of NatA, what stabilizes SNC1, induces resistance to *Hyaloperonospora arabidopsidis* Noco2 and *Pseudomonas maculicola*, whereas disruption of NatB activity has no visible effect (Xu et al. 2015).

BIOTECHNOLOGICAL AND CLASSICAL BREEDING APPROACHES TO ADDRESS THE USE OF N-DEGRON PATHWAYS AND SUBSTRATES FOR PLANT IMPROVEMENT AND NOVEL APLICATIONS

Because the plant N-degron pathways modulate the stability of different cohorts of substrates they have great potential for biotechnological applications, including molecular farming, and for classical breeding approaches to enhance plant performance in crop systems. Manipulation of the stability of specific substrates in relation to N-degron pathway regulation may also hold great promise. Examples of this already exist, as loci encoding SUB1A and SK1/2 ERFVIIs are already used in breeding programmes to produce rice types with improved flood-tolerance, stabilising yields against unpredictable weather (Bailey-Serres et al. 2012). An important discovery is that arabidopsis *prt6* mutant plants show improved tolerance to a wide variety of stresses, whilst not impacting greatly on normal development, indicating that manipulation of N-recognin function could be a useful approach to enhance plant performance. Manipulation of *PRT6* expression or function through transgenesis or classical mutagenesis in barley demonstrated an increased tolerance of this crop to extremes of water (waterlogging and flooding) and salinity, and improved tolerance towards some pathogens (Mendiondo et al. 2016; Vicente et al. 2017; Vicente et al. 2019). The PRT6/ERFVII-regulated response of Poplar to excess zinc also suggests possible approaches to improve plant zinc-tolerance (Dalle Carbonare et al. 2019). These results indicate that manipulation of this pathway may be one productive avenue towards delivery of ‘climate-change ready’ crops (Varshney et al. 2018).

The ability to manipulate the stability of specific N-degron pathway substrates has great promise for biotechnological applications to produce ‘phenotypes on demand’ (Faden et al. 2014) that depend on the ability to dictate the time and place of changed stability and downstream consequences. As an example of this approach in plants, the ubiquitin-fusion-technique was used to produce a temperature-sensitive version of DHFR (Dihydrofolate reductase; DHFR T39A,E173D) with Nt-Phe (therefore a target for PRT1) linked to the transcription factor TRANSPARENT TESTA GLABRA (TTG)1, that positively regulates the development of trichomes on the leaf surface (Faden et al. 2014). The Nt-Phe-DHFR-TTG1 proteoform was stable at both restrictive (high) and permissive (low) temperatures in the *prt1*

mutant compared to wild-type, demonstrating the necessity of the N-degron for degradation, and in the *ttg1* mutant background temperature-dependent development of trichomes was observed (Figure 2H). In a second example using the flowering time regulator CONSTANS (CO), early induction of flowering was achieved (Faden et al. 2014). This system was also used to direct conditional expression of the highly cytotoxic RNase barnase, providing a switchable mechanism to regulate cell fate (Faden et al. 2019). Other synthetic biology approaches have been used to reconstitute the plant-specific PCO-dependent components of the PRT6 pathway in yeast (Puerta et al. 2019) and to provide tools to assess in vivo Nt-Arg and Nt-Phe PRT6/PRT1 N-degron pathway substrate half-life with Tandem fluorescent protein timers (Zhang et al. 2019). These advances indicate the potential use of N-degron mediated regulation of protein stability for production and manipulation of proteins of biotechnological interest, or for metabolic engineering.

FUTURE PROSPECTS FOR UNDERSTANDING AND DISCOVERING N-DEGRON PATHWAY COMPONENTS, SUBSTRATES AND FUNCTIONS IN PLANT BIOLOGY

Known and unknown plant N-degron pathways and links to other mechanisms regulating proteostasis

Currently knowledge of plant N-degron pathways trails work in other eukaryotic kingdoms. Plant versions of fMet/, Pro/ and Gly/ N-degron pathways have not been investigated, putative Ac/N-degron pathway components require investigation and validation, and known destabilizing residues of UBR1 are not targets of PRT6 or PRT1 (Figure 1A). Thus, more work will be needed to fully understand the reach of N-degron pathways in terms of substrate recognition. Whilst there are a growing number of physiological roles assigned for the PCO and NTAQ1 branches of the PRT6 pathway, NTAN1, ATE and E3 ligase (PRT6 and PRT1)-specific roles remain to be elucidated, as do the identification of physiological substrates for most of these branches.

It is likely that plant N-degron pathways are integrated into other cellular mechanisms of proteostasis. A possible link between the Arg/N-degron pathway and Nt-acetylation was recently identified in yeast, where it was suggested that acetylation of N-termini with destabilizing residues may protect proteoforms from selection for degradation by UBR1 (Kats et al. 2018), and it is possible that this may also occur in plants. Autophagy is a major mechanism for removal of unwanted intracellular proteins (Liu and Bassham 2012), where cargo adaptors bind material that is transported to autophagosomes. Recently a direct link between N-degron pathways and autophagy was shown in mammals to regulate Nt-arginylation by ATE of several endoplasmic reticulum chaperone proteins and subsequent interaction with the autophagic adaptor p62, leading to autophagic degradation (Cha-Molstad

et al. 2015). Whether a similar mechanism might operate in plants remains unclear, however, it has been shown that hypoxia induces autophagy and mutants of the autophagy pathway are more susceptible to submergence (Chen et al. 2015), that might indicate an interaction between the PRT6 N-degron pathway and autophagy. In addition to N-degron pathways, recently degradation dependent on the Ct- of proteoforms was discovered using combinations of global protein stability profiling and a synthetic human peptidome (Koren et al. 2018; Lin et al. 2018). C-end degrons of truncated or altered proteins formed as a consequence of altered synthesis, folding or stress are targeted by a C-degron mechanism (DesCEND) for degradation. The C-degrons minimally contain 6-10 residues at the Ct-, and substrate recognition occurs through various interchangeable substrate adapters by diverse Cullin-RING E3 ligase complexes. It is very tempting to speculate that a similar mechanism exists in plants, particularly as target sites in substrates of endopeptidases might reveal C-degron compatible residues (Tsiatsiani et al. 2013).

In or out? Protease action, variation in substrate stability and ecological functions

Key to the function of N-degrons is the activity of endoproteases in initiating entry into N-degron pathways. There is likely to be biological meaning to the coupling of protease expression and activity with downstream expression of target proteins and components of N-degron pathways. Understanding how the matching of these two different elements results in the regulated stability of proteoforms with destabilising residues will be an important goal of future research in this area. In addition, new methodologies for N-terminomics linked to specific proteases will allow a expansion in the availability of putative N-degron pathway substrates for investigation. Determination of in-vivo half-lives of substrates will be important and methodologies are being developed to address this. Tandem fluorescent timers (tFTs) to study protein turnover rates in vivo have recently been developed (Zhang et al. 2019), using a fusion of two single colour fluorescent proteins (FP) that undergo fluorophore maturation with different kinetics. The ratio of slow-maturing FP to fast-maturing FP fluorescence intensities provides a measure of protein age through a single time point image. This approach was used to show the destabilising capacity of Nt-residues dependant on N-recognin activity in vivo in plants. Thought needs to be given to understanding the relative stabilities of N-degron pathway substrates, and the effect of this on physiology. For example, although a molecular explanation for how SUB1A evades N-degron pathway degradation has been defined (Lin et al. 2019), the role of N-degrons in regulating the stability of the rice Met-Cys initiating ERFVIIs SK1 and SK2 has not been investigated. It would certainly be interesting to understand whether these proteins also evade degradation via Cys2, or whether they are regulated by different pathways. More generally it is possible to speculate that the N-degron pathway regulated stability of ERFVIIs (and other substrates) in different species may vary

depending on their eco-physiology, that may present useful possibilities for general improvement of environmental stress tolerance in crops.

From a slow start, we have come a very long way very quickly in understanding the roles of N-degron pathways in plants. It is clear that there is much more to be discovered in this fascinating area that links the amino-terminal residue of a protein to protease action and proteostasis, resulting in the regulation of fundamental aspects of plant growth and development, and environmental interactions.

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AUTHOR CONTRIBUTIONS

M.J.H. conceived the idea and prepared the manuscript with J.V, G.S. and A.Z. GS contributed the images of RAP2.12 in Figure 2D.

FIGURES AND TABLES:

Figure 1: Overview of plant N-degron pathways

A. Schematic representation of plant N-degron pathways. Cleavage of proteins by exo- or endo-peptidases leads to the production of novel Ct-proteoforms that may have destabilising residues. The identity of primary, secondary and tertiary destabilising residues is shown. The single amino-acid code is used. fMet, formyl-Met, ^{ac}X, Nt-acetylated residue. **Φ** denotes hydrophobic residues; C^{OX}, Cys-sulfinic acid; PCO, Plant Cysteine Oxidase; NTAQ1, Nt-Gln amidase; NTAN1, Nt-Asn amidase; MetAP, Methionine amino-peptidase. The functional position of NO in the PCO branch of the PRT6 N-degron pathways is not currently known.

B, C. Domain structures of plant known (bold) and putative N-recognins related the Arg/N-degron pathway; PRT6 and PRT1 and yeast UBR1 N-recognins, and putative plant Ac/N-recognins related to yeast Doa10/Not4.

Relative sizes are shown, with functional domains highlighted. Different Zinc finger domains-Really Interesting New Gene (RING), ZZ and PHD; AI, autoinhibition domain; UBR, UBR box domain; bacterial ClpS-like motif; RRM, RNA recognition domain.

Figure 2: Plant N-degron pathways and substrates influence many aspects of growth, development and environmental interactions

A. Enhanced tolerance of barley plants containing two different alleles of *HvPRT6* to waterlogging compared to wild-type plants (images taken from (Mendiondo et al. 2016)).

B. Enhanced survival of arabidopsis seedlings following extended skotomorphogenic development in hypoxia, compared to normoxia, when subsequently exposed to normoxia in the light, is controlled by ERFVII transcription factors (images taken from (Abbas et al. 2015)). All seedlings except wild-type (WT) grown under hypoxia in the dark are dead.

C. NO gas represses hypocotyl elongation of skotomorphogenic seedlings, that is controlled by ERFVII transcription factors RAP2.12, RAP2.2 and RAP2.3 in a *PRT6* dependent manner (images taken from (Gibbs et al. 2014b)).

D. Meristem of arabidopsis contain a hypoxic niche that stabilises substrates of the PCO branch of the PRT6 N-degron pathway, including ZPR2 (from *promZPR2:ZPR2-GUS*), VRN2 (from *promVRN2:VRN2-GUS*) (image taken from (Gibbs et al. 2018)) and RAP2.12 (from *promRAP2.12:RAP2.12-GUS*). In each case proteins are expressed under their own promoters (prom) and Ct-tagged with the GUS reporter protein, allowing histochemical localisation of stabilised protein. Whereas *promZPR2* drives expression in the SAM, *VRN2* and *RAP2.12* RNAs are expressed throughout development at high levels (for example, compare VRN2-GUS staining in *prt6* and Col-0).

E. Barley plants with reduced expression of *HvPRT6* (*HvPRT6* RNAi) are more tolerant of watering with salt than non-transgenic null segregant plants (images taken from (Vicente et al. 2017)).

F. The stability of ERFVII RAP2.12 is enhanced in normoxia in the presence of ethylene. Confocal imaging and quantification of RAP2.12-GFP in arabidopsis root tips incubated in air or ~5$\mu l l^{-1}$ ethylene (images taken from (Hartman et al. 2019)).

G. ERFVII transcription factor stability, controlled by the ATE branch of the PRT6 N-degron pathway enhances clubroot symptoms on arabidopsis plants (image taken from (Gravot et al. 2016)).

H. Biotechnological use of the PRT1 N-degron pathway; induction of trichomes in the *ttg1* mutant (that lack trichomes) via constitutive expression of a temperature-sensitive PRT1-regulated N-degron F-DHFR(T39A,E173D)-TTG1 (*prom35S:K2:TTG1*). Trichomes are formed at the lower permissive temperature because the degron is non-functional. Arrow heads indicate developed trichomes. Scale bars are 1 cm, 5 mm (close-up) (images taken from (Faden et al. 2016)).

Table 1: Physiological roles of N-degron pathway components and substrates

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