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Homeostatic response to hypoxia is regulated by the N-end rule pathway in plants

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Abstract

Plants and animals are obligate aerobes, requiring oxygen for mitochondrial respiration and energy production. In plants, an unanticipated decline in oxygen availability (hypoxia), as caused by root waterlogging or foliage submergence, triggers changes in gene transcription and mRNA translation that promote anaerobic metabolism and thus sustain substrate-level ATP production[1]. In contrast to animals[2], oxygen sensing has not been ascribed to a mechanism of gene regulation in response to oxygen deprivation in plants. Here we show that the N-end rule pathway of targeted proteolysis acts as a homeostatic sensor of severe low oxygen in *Arabidopsis*, through its regulation of key hypoxia response transcription factors. We found that plants lacking components of the N-end rule pathway constitutively express core hypoxia response genes and are more tolerant of hypoxic stress. We identify the hypoxia-associated Ethylene Response Factor (ERF) Group VII transcription factors of *Arabidopsis* as substrates of this pathway. Regulation of these proteins by the N-end rule pathway occurs through a characteristic conserved motif at the N-terminus initiating with MetCys- (MC-). Enhanced stability of one of these proteins, HRE2, under low oxygen conditions improves hypoxia survival and reveals a molecular mechanism for oxygen sensing in plants via the evolutionarily conserved N-end rule pathway. SUB1A-1, a major determinant of submergence tolerance in rice[3], was shown not to be a substrate for the N-end rule pathway despite containing the N-terminal motif, suggesting that it is uncoupled from N-end rule pathway regulation, and that enhanced stability may relate to the superior tolerance of Sub1 rice varieties to multiple abiotic stresses[4].

The N-end rule pathway of targeted proteolysis associates the fate of a protein substrate with the identity of its N-terminus (the N-degron)[5,6]. The N-terminal residue is classified as stabilising or destabilising, depending on the fate of the protein. An N-degron containing a

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destabilising residue is created through specific proteolytic cleavage, but can also be generated via successive enzymatic or chemical modifications to the N-terminus, for example, arginylation by Arg-tRNA protein transferases (ATE)[7,8,9] (Supplementary Fig.1). N-end rule pathway substrates containing destabilising residues are targeted for proteasomal degradation via specific E3 ligases (also known as N-recognins), such as PROTEOLYSIS1 and 6 (PRT1 and 6) in *Arabidopsis*, which accept substrates with hydrophobic and basic amino termini, respectively[8,9,10]. Several substrates of the N-end rule pathway are important developmental regulators in mammals[11] but as yet no substrates have been identified in plants. Previously we showed a function of this pathway in Abscisic Acid (ABA) signalling through PRT6 and ATE[12], and it has also been associated with leaf senescence and shoot and leaf development[13,14] in *Arabidopsis*. To understand N-end rule pathway-regulated gene expression we analysed the transcriptome of imbibed seed and seedlings of N-end rule pathway mutants *prt6* and *ate1 ate2*, which lacks ATE activity[14] (Fig. 1a; Supplementary Table 1). This analysis revealed that genes important for anaerobic metabolism and survival of hypoxia, such as *ADH1, SUS4* and *PDC1* were constitutively expressed at high levels in both mutants, in common with wild-type (WT) Col-0 plants under hypoxia (Supplementary Fig. 2). For example, 47 of the 135 differentially regulated mRNAs in the WT hypoxia-induced transcriptome were also up-regulated in *prt6* seedlings grown under non-stress conditions (Supplementary Table 1; signal $\log_2$ ratio ≥1, False Discovery Rate ≤ 0.01). The *ptr6* and *ate1 ate2* up-regulated mRNAs included over half of the core 49 mRNAs up-regulated by hypoxia across seedling cell types[15] (Fig. 1b; Supplementary Fig. 2). Consistent with this observation ß-glucuronidase (GUS) expression driven by the promoter of *ADH1* (*pADH1::GUS*[16]) was up-regulated in WT seedlings subjected to hypoxia and ectopically expressed in mature embryos, roots and lower hypocotyls of *prt6* (Fig. 1c, Supplementary Fig. 3). Constitutive expression of hypoxia-induced genes by N-end rule pathway mutant seedlings suggested that they would be resistant to hypoxic conditions. Imbibed seeds of both *prt6* and *ate1 ate2* were able to germinate well under low oxygen (3%) compared to WT (Fig. 1d), and mutant seedlings were more able to survive prolonged oxygen deprivation (Fig. 1e,f). The *ate1 ate2* double mutant showed greater resistance to hypoxia than *prt6* suggesting the existence of other as yet unidentified Arg-related E3 ligases as previously postulated[10,14].

Transcription factors of the five member *Arabidopsis* Ethylene Response Factor (ERF) Group VII[17] have recently been shown to enhance plant responses to hypoxia or anoxia, including HYPOXIA RESPONSIVE(HRE)1 and 2[18] and RELATED TO AP2(RAP)2.2[19]. Over-expression of RAP2.12 was also shown to induce expression of a *pADH1::LUCIFERASE* reporter gene[20]. This subfamily shows homology to the agronomically important rice ERFs SUBMERGENCE(SUB)1A, B, C[3] and SNORKEL1 and 2[21]. *SUB1A-1* within the *SUBMERGENCE1* (*SUB1*) locus (which also contains *SUB1B* and *SUB1C*) was shown to be a primary determinant of enhanced survival of rice plants under complete submergence[3]. With the exception of SUB1C, all contain the initiating motif MC- at the N-terminus, embedded within a longer consensus shared with most other Group VII ERFs of *Arabidopsis* and rice, MCGGAII (Supplementary Fig. 4a).

Removal of N-terminal methionine by METHIONINE AMINO-PEPTIDASE(MAP) reveals the tertiary destabilising residue cysteine in proteins initiating with MC-, which targets substrates for degradation by the N-end rule pathway[7,9,22] (Supplementary Fig.1). In mouse, N-end rule pathway-mediated degradation of the MC-motif containing G-protein signalling components RGS4 and RGS5 is perturbed under hypoxia[22,23]. It was hypothesised that oxidation of C2 (Cys at position 2) in these proteins under normoxia creates a secondary destabilising residue allowing addition of Arg (R) to the N-terminus by ATE, creating a primary destabilising residue[23]. We investigated the possibility that all *Arabidopsis* Group VII ERFs as well as rice SUB1A-1 are N-end rule pathway substrates. A heterologous rabbit

reticulocyte lysate assay[23] was used to express ERFs driven by a T7 promoter *in vitro*, because components of the N-end rule pathway (ATE, MAP and PRT6) are highly conserved in eukaryotes[8], and it has been shown that wheat-germ lysate does not contain an active proteosomal system[24]. *Arabidopsis* Group VII ERFs were short-lived, and their stability was enhanced by MG132 and the N-end rule pathway competitive dipeptide Arg-β-Ala but not by the non-competitive Ala-Ala dipeptide[23] (Fig. 2a). Mutation of C2 to Ala (A) (^{C2A}), which should remove the N-degron and stabilise proteins specifically with respect to the N-end rule pathway[23], significantly enhanced stability *in vitro* of *Arabidopsis* ERFs, suggesting that all Group VII ERFs are potential substrates of the N-end rule pathway. *Arabidopsis* contains 206 proteins from gene models with MC- at the N-terminus; we used two of these, VERNALISATION(VRN)2 and MADS AFFECTING FLOWERING(MAF)5, which lack the extended N-terminal Group VII ERF consensus (Supplementary Fig. 4b), to test specificity of this sequence. Whereas VRN2-HA was degraded in this system, and stabilised by the introduction of a C2A mutation (VRN2^{C2A}-HA), MAF5-HA and MAF5^{C2A}-HA were both stable (Fig. 2b), indicating that not all *Arabidopsis* MC- proteins are N-end rule pathway substrates. This is not surprising as it has previously been shown that optimal positioning of a downstream lysine for ubiquitination is also a key determinant of the quality of an N-degron[8,9,25]. SUB1A-1 was resistant to degradation (Fig. 2c). As the N-terminal sequence of SUB1A-1 differs at position 5 (E rather than A, Supplementary Fig. 4a) we analysed a mutant version that replaced this amino acid to reconstitute the consensus Group VII sequence (Sub1A^{E5A}). Sub1A^{E5A} was also stable *in vitro* (Fig. 2c), suggesting that degradation of this protein is uncoupled from the N-end rule pathway. As expected, the rice protein SUB1C, lacking an MC N-terminus, was long lived *in vitro* (Fig. 2c).

To confirm activity of the N-end rule pathway towards specific MC-containing substrates in plants, we analysed the *in vivo* longevity of the ERF proteins HRE1 and 2 (Fig. 2d). We expressed either WT or mutant (HRE1^{C2A}, HRE2^{C2A}) HA-tagged versions of these proteins ectopically using the CaMV35S promoter in *Arabidopsis*. In WT plants, only the mutant C2A proteins could be detected at high levels, despite detectable expression of corresponding mRNAs, suggesting that WT versions are N-end rule pathway substrates *in vivo*. HRE2-HA expressed in the *prt6* mutant was stable, linking its degradation directly to PRT6. To assess whether oxygen regulates the stability of HRE proteins, we analysed the accumulation of HRE-HA proteins in WT plants expressing HRE1-HA, HRE1^{C2A}-HA, HRE2-HA and HRE2^{C2A}-HA under normal and low oxygen conditions (Fig. 3a). Following transfer of seedlings to hypoxic conditions we observed elevation of HRE2-HA within 2 hours, but could not detect HRE1-HA (Fig. 3a; Supplementary Fig. 5a,b). HRE2-HA became destabilised again upon return to normoxic conditions (Fig. 3a). Both seeds and seedlings ectopically expressing stable C2A versions of HRE1 and HRE2 had increased tolerance to extended periods of oxygen deprivation (Fig. 3b,c,d; Supplementary Fig. 5c).

These data demonstrate that *Arabidopsis* ERF Group VII transcription factors are substrates of the N-end rule pathway, and function to sense molecular oxygen, most likely through oxidation of the tertiary destabilising residue cysteine. Stabilisation of these proteins under hypoxic conditions leads to increased survival under low oxygen stress (Fig. 3e). It is currently unclear whether oxidation occurs through a chemical or enzymatic mechanism, although cysteine is readily oxidised chemically[26]. It is also unclear whether oxidation is related directly to molecular oxygen, or if indirect cellular changes associated with oxygen availability (such as alterations in cytosolic pH[27] and specific metabolites or transient accumulation of reactive oxygen species[1]) might trigger cysteine oxidation. SUB1A-1 may provide enhanced responsiveness to submergence and drought in rice in part due to the fact that it is not a substrate of the N-end rule pathway. By contrast, the condition-dependent destabilization of group VII ERFs in Arabidopsis could require oxygen levels to decline below some threshold before these factors can activate anaerobic gene transcription. It is

probable SUB1A-1 evades the N-end rule pathway due to the absence of an optimally positioned lysine downstream of the N-degron, since substrate quality is determined combinatorially by an N-degron destabilising residue and downstream lysine position[8,9,25]. Alternatively differences in protein tertiary structure may preclude N-terminus accessibility. SUB1A-1 was also recently shown to mediate crosstalk between submergence and drought tolerance in rice by augmenting ABA responsiveness[4], suggesting a link between drought tolerance and the previously identified function of the N-end rule pathway in removing responsiveness to ABA[12]. Targeted degradation of proteins by the N-end rule pathway was identified as a homeostatic mechanism in mammalian systems[22,23,28], for example in the control of hypoxia-related expression of RGS4[28] and RGS5[23]. It is fascinating that the N-end rule pathway carries out the same functionality in relation to low oxygen stress in plants, but taking as substrates members of a plant specific transcription factor family. This highlights evolutionary conservation of the mechanism of oxygen perception across kingdoms using the N-end rule pathway independent of the targets. Our confirmation of *in vivo* function of two members of the ERF Group VII sub-family provides direct evidence for the control of HRE2 by oxygen and the N-end rule pathway and indirect evidence that HRE1 is also a N-end rule pathway substrate *in vivo*. We demonstrate that all members of *Arabidopsis* Group VII ERFs are N-end rule pathway substrates *in vitro*, and thus it is possible that all members orchestrate N-end rule pathway-controlled, hypoxia-related functions. Identification and manipulation of N-end rule pathway substrates will therefore be a key target for both conventional breeding and biotechnological approaches in relation to manipulation of plant responses to abiotic stress.

Methods Summary

Protein stability analyses

Full length cDNAs were PCR amplified from either *Arabidopsis thaliana* or *Oryza sativa* L. (cv. M202(Sub1)). N-terminal mutations were introduced using the forward primer (Supplementary Table 2). For *in vitro* assays, cDNAs were cloned into a modified version of the pTNT vector (Promega) to produce C-terminal HA fusions. Stability assays were performed using the TNT T7 Coupled Reticulocyte Lysate system (Promega), essentially as described previously[23]. For *in vivo* analysis of HRE-HA proteins, cDNAs were cloned into pE2c, mobilised into pB2GW7 and transformed into *Arabidopsis* using the floral dip method. To assess relative protein stability, equal amounts of total protein extracted from 7-day old T_3 homozygous seedlings were analysed by Western blot, and cDNA synthesised from total RNA was used as a template for semi-quantitative PCR.

Gene expression analyses

For microarray analysis, total RNA extracted from seeds[12] or seedlings[15] was hybridised against the *Arabidopsis* ATH1 genome array (Affymetrix). Differentially expressed genes were clustered as described previously[15]. *pADH::GUS*[16] was crossed to *prt6-1* and homozygous seeds or seedlings were analysed for GUS activity before and after submergence for the times indicated.

Low O_2 phenotypic analyses

To assess germination (scored as radicle emergence), imbibed seeds were incubated for 7-days in chambers flushed with varying O_2 tensions[29]. For 7-day old seedling survival, O_2 deprivation was achieved by bubbling 99.995% Argon through water into chambers under positive pressure, before recovering in air for 3 days and scoring of plants (n=15) per plate that were non-damaged, damaged or dead (scored 5, 3 and 1, respectively)[15]. The same argon chambers were used to treat seedlings for the times indicated prior to protein extraction for Western blot analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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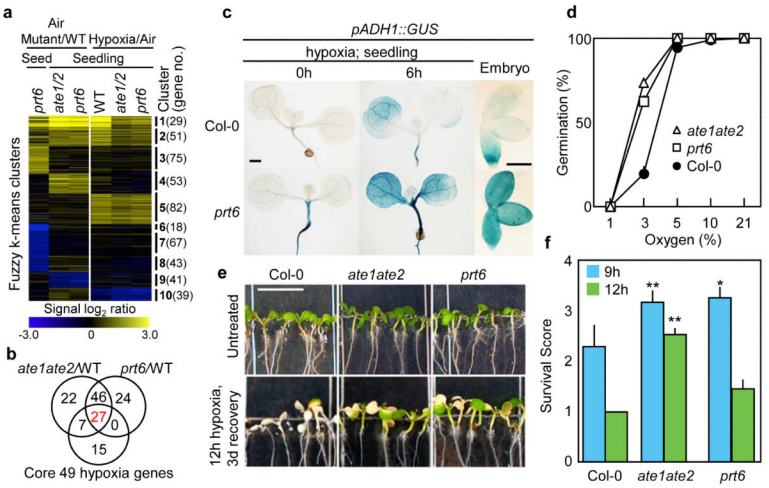

Figure 1. N-end rule mutants ectopically accumulate anaerobic response mRNAs and are more tolerant to hypoxia

a. Expression data for differentially expressed genes comparing WT (Col-0) and mutants under air or hypoxia (2 h $-O_2$).

b. mRNAs upregulated in mutants overlap with 49 mRNAs induced across cell types by hypoxia in WT seedlings[15].

c. Spatial visualization of *ADH1* promoter activity. Scale bars 100 μm.

d. Germination under reduced oxygen availability.

e. Seedlings after 12 h of hypoxia and 3 d recovery. Scale bar 0.6 cm.

f. N-end rule pathway mutants are less sensitive to hypoxia stress. Data are mean of replicate experiments ± SD; * = $P < 0.05$, ** = $P < 0.01$.

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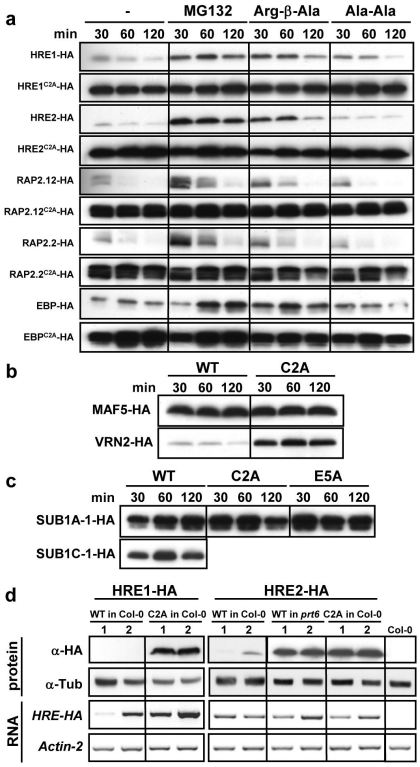

Figure 2. Group VII ERF transcription factors are substrates for the N-end rule pathway *in vitro* and *in vivo*

a. Western blot analysis of *in vitro* stability of HA-tagged WT and C2A variants of *Arabidopsis* Group VII ERFs in the absence or presence of MG132, N-end rule pathway competitive dipeptide (Arg-β-Ala) or noncompetitive dipeptide (Ala-Ala).

b. *In vitro* stability of WT and C2A VRN2-HA and MAF5-HA.

c. *In vitro* stability of HA-tagged rice ERFs.

d. *In vivo* protein stability and RNA expression levels of WT and C2A variants of HRE1-HA and HRE2-HA ectopically expressed in *Arabidopsis*, shown for two independent transformed lines (1 and 2).

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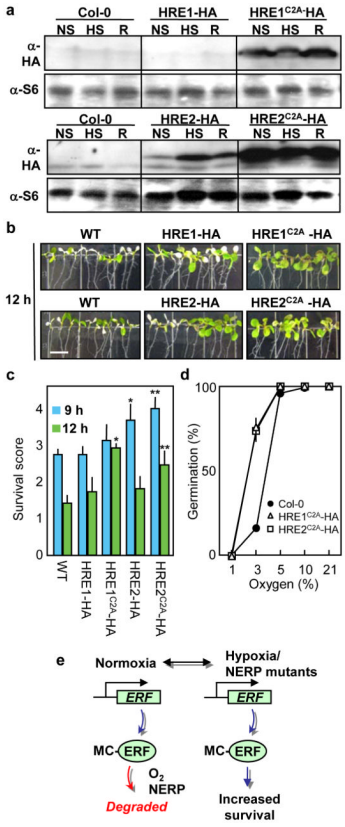

Figure 3. HRE proteins are stabilized under low oxygen and confer hypoxia tolerance

a. *In vivo* stability of WT and C2A HRE1-HA, HRE2-HA (α-HA) or S6 (Ribosomal protein S6) control (α-S6): no stress (NS), 2 h hypoxia (HS), following 1 h recovery from stress (R).

b. Seedlings expressing WT or C2A HRE1-HA and HRE2-HA after 12 h hypoxic stress and 3 d of recovery. Scale bar = 0.6 cm.

c. Seedling survival for WT or C2A HRE1-HA and HRE2-HA after 9 h or 12 h hypoxic stress. Data are mean of replicate experiments ± SD; * = $P < 0.05$, ** = $P < 0.01$.

d. Germination under reduced oxygen availability.

e. Model explaining N-end rule pathway mediated oxygen-dependent turnover of Group VII ERFs in *Arabidopsis*.

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