LETTER

doi:10.1038/nature10534

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

Daniel J. Gibbs[1]*, Seung Cho Lee[2]*, Nurulhikma Md Isa[1], Silvia Gramuglia[1], Takeshi Fukao[2], George W. Bassel[1], Cristina Sousa Correia[1], Françoise Corbineau[3], Frederica L. Theodoulou[4], Julia Bailey-Serres[2] & Michael J. Holdsworth[1]

**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 roots becoming waterlogged or foliage submergence, triggers changes in gene transcription and messenger RNA 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 levels 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 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 amino terminus initiating with Met-Cys. 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, indicating 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 stabilizing or destabilizing, depending on the fate of the protein. An N degron containing a destabilizing 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)[7–9] (Supplementary Fig. 1). N-end rule pathway substrates containing destabilizing residues are targeted for proteasomal degradation via specific E3 ligases (also known as N recognins), such as PROTEOLYSIS1 and PROTEOLYSIS6 (PRT1 and PRT6) in *Arabidopsis*, which accept substrates with hydrophobic and basic N termini, respectively[8–10]. 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 in *Arabidopsis*[13,14]. To understand N-end-rule-pathway-regulated gene expression we analysed the transcriptome of imbibed seed and seedlings of N-end rule pathway mutants *ate1 ate2*, which lack ATE activity[14], and *prt6* (Fig. 1a and 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 Col-0 plants under hypoxia (Supplementary Fig. 2). For example, 47 of the 135 differentially regulated mRNAs in the wild-type hypoxia-induced transcriptome were also upregulated in *prt6* seedlings grown under non-stress conditions (Supplementary Table 1; signal $\log_2$ ratio $\geq 1$, false discovery rate $\leq 0.01$). The mRNAs upregulated in *prt6* and *ate1 ate2* mutants included over half of the core 49 mRNAs upregulated by hypoxia across seedling cell types[15] (Fig. 1b and Supplementary Fig. 2). Consistent with this observation, β-glucuronidase (GUS) expression driven by the promoter of *ADH1* (*pADH1::GUS*; ref. 16) was upregulated in wild-type seedlings subjected to hypoxia and ectopically expressed in mature embryos, roots and lower hypocotyls of *prt6* mutants (Fig. 1c and 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* mutants were able to germinate well under low oxygen (3%) compared to wild type (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*, indicating 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 RESPONSIVE1 and 2 (HRE1 and HRE2)[18] and RELATED TO AP2 2 (RAP2.2)[19]. Overexpression 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 1A, B and C (ref. 3) and SNORKEL 1 and 2 (ref. 21). *SUB1A-1* within the *SUBMERGENCE 1* (*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 Met-Cys (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 AMINOPEPTIDASE (MAP) reveals the tertiary destabilizing 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 hypothesized that oxidation of cysteine at position 2 (C2) in these proteins under normoxia creates a secondary destabilizing

[1]Division of Plant and Crop Sciences, School of Biosciences and Centre for Plant Integrative Biology, University of Nottingham, Loughborough LE12 5RD, UK. [2]Center for Plant Cell Biology and Department of Botany and Plant Sciences, University of California, Riverside, California 92521, USA. [3]UPMC Univ Paris 06, UR5-EAC 7180 CNRS, Boîte courrier 156, 4 place Jussieu, F-75005 Paris, France. [4]Biological Chemistry Department, Rothamsted Research, Harpenden AL5 2JQ, UK.
*These authors contributed equally to this work.

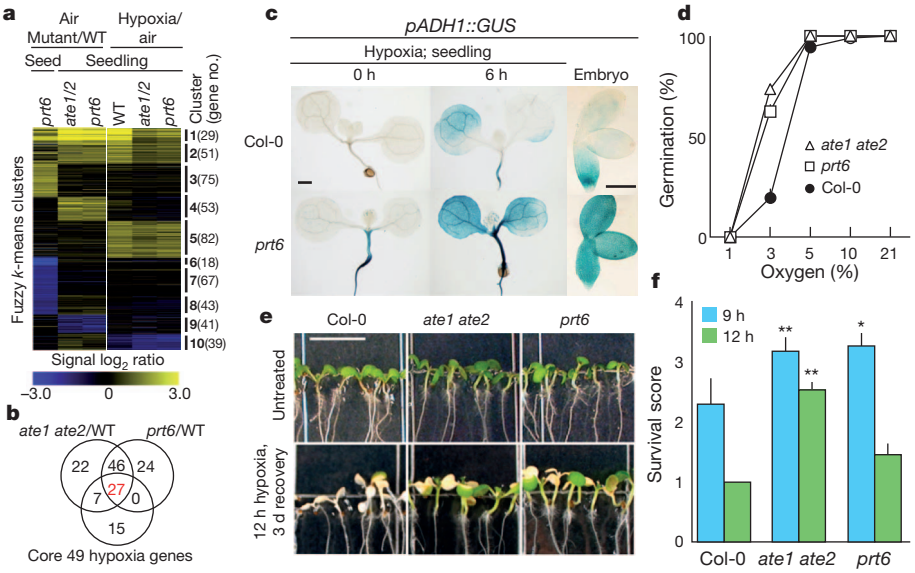


**Figure 1 | N-end rule mutants ectopically accumulate anaerobic response mRNAs and are more tolerant to hypoxia.** **a**, Expression data for differentially expressed genes comparing wild-type (Col-0) and mutants under air or hypoxia (2 h $-O_2$). **b**, mRNAs upregulated in mutants overlap with 49 mRNAs induced across cell types by hypoxia in wild-type 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 ± s.d.; $*P < 0.05$; $**P < 0.01$.

residue allowing addition of arginine (R) to the N terminus by ATE, creating a primary destabilizing 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 haemagglutinin (HA)-tagged 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 alanine (C2A), which should remove the N-degron and stabilize proteins specifically with respect to the N-end rule pathway[23], significantly enhanced stability *in vitro* of *Arabidopsis* ERFs, indicating 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 2 (VRN2) and MADS AFFECTING FLOWERING 5 (MAF5), which lack the extended N-terminal group VII ERF consensus (Supplementary Fig. 4b)—to test the specificity of this sequence. Whereas HA-tagged VRN2 (VRN2–HA) was degraded in this system, and stabilized 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-1(E5A)–HA). SUB1A-1(E5A)–HA was also stable *in vitro* (Fig. 2c), indicating that degradation of this protein is uncoupled from the N-end rule pathway. As expected, the rice protein SUB1C-1–HA, lacking an MC N terminus, was long lived *in vitro* (Fig. 2c).

To confirm the 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 HRE2 (Fig. 2d). We expressed either wild-type or mutant (HRE1(C2A), HRE2(C2A)) HA-tagged versions of these proteins ectopically using the CaMV35S promoter in *Arabidopsis*. In wild-type plants, only the mutant C2A proteins could be detected at high levels, despite detectable expression of corresponding mRNAs, indicating that wild-type 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 wild-type plants expressing HRE1–HA, HRE1(C2A)–HA, HRE2–HA and HRE2(C2A)–HA under normal and low oxygen conditions (Fig. 3a). After transfer of seedlings to hypoxic conditions we observed elevation of HRE2–HA within 2 h, but could not detect HRE1–HA (Fig. 3a and Supplementary Fig. 5a, b). HRE2–HA became destabilized 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–d and 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 destabilizing residue cysteine. Stabilization 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 oxidized 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 that SUB1A-1 evades the N-end rule pathway due to the absence of an optimally positioned lysine downstream of the N degron, as substrate quality is determined combinatorially by an N degron destabilizing residue and downstream lysine position[8,9,25]. Alternatively, differences in protein tertiary structure may preclude

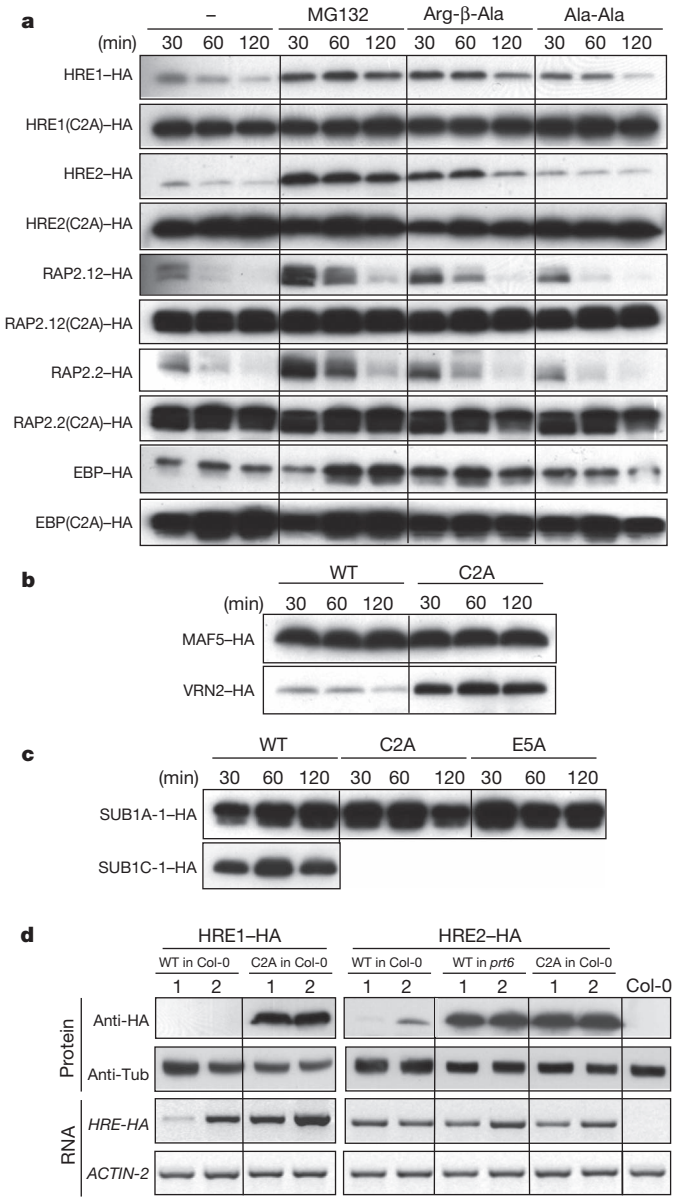


**Figure 2 | Group VII ERF transcription factors are substrates for the N-end rule pathway *in vitro* and *in vivo*.** **a**, Western blot analysis of *in vitro* stability of HA-tagged wild-type and C2A variants of *Arabidopsis* group VII ERFs in the absence or presence of MG132, N-end rule pathway competitive dipeptide (Arg-β-Ala) or non-competitive dipeptide (Ala-Ala). **b**, *In vitro* stability of wild-type 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 wild-type and C2A variants of HRE1–HA and HRE2–HA ectopically expressed in *Arabidopsis*, shown for two independent transformed lines (1 and 2).

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 (ref. 28) and RGS5 (ref. 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 ERF

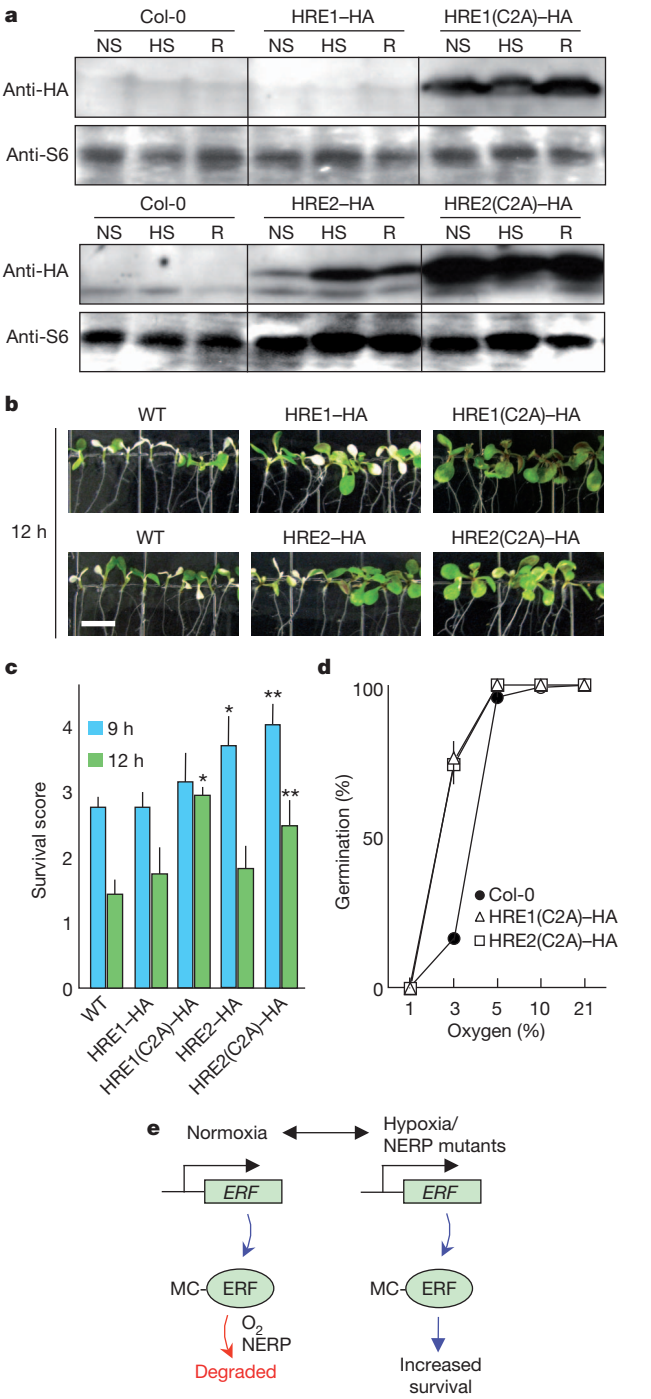


**Figure 3 | HRE proteins are stabilized under low oxygen levels and confer hypoxia tolerance.** **a**, *In vivo* stability of wild-type and C2A HRE1–HA, HRE2–HA (anti-HA) or S6 (ribosomal protein S6) control (anti-S6). HS, 2 h hypoxia; NS, no stress; R, following 1 h recovery from stress. **b**, Seedlings expressing wild-type 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 wild-type or C2A HRE1–HA and HRE2–HA after 9 h or 12 h hypoxic stress. Data are mean of replicate experiments ± s.d. $*P < 0.05$; $**P < 0.01$. **d**, Germination under reduced oxygen availability. **e**, Model explaining N-end-rule-pathway-mediated oxygen-dependent turnover of group VII ERFs in *Arabidopsis*.

group VII provides direct evidence for the control of HRE2 by oxygen and the N-end rule pathway and indirect evidence that HRE1 is also an 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 amplified by polymerase chain reaction (PCR) 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, mobilized 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 synthesized 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 hybridized 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 before protein extraction for western blot analysis.

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

**Received 13 June; accepted 5 September 2011.**
**Published online 23 October 2011.**

1. Bailey-Serres, J. & Voesenek, L. A. C. J. Flooding stress: Acclimations and genetic diversity. *Annu. Rev. Plant Biol.* **59,** 313–339 (2008).
2. Kaelin, W. G. & Ratcliffe, P. J. Oxygen sensing by metazoans: The central role of the HIF hydroxylase pathway. *Mol. Cell* **30,** 393–402 (2008).
3. Xu, K. *et al. Sub1A* is an ethylene-response-factor-like gene that confers submergence tolerance to rice. *Nature* **442,** 705–708 (2006).
4. Fukao, T., Yeung, E. & Bailey-Serres, J. The submergence tolerance regulator SUB1A mediates crosstalk between submergence and drought tolerance in rice. *Plant Cell* **23,** 412–427 (2011).
5. Bachmair, A., Finley, D. & Varshavsky, A. *In vivo* half-life of a protein is a function of its amino-terminal residue. *Science* **234,** 179–186 (1986).
6. Varshavsky, A. Regulated protein degradation. *Trends Biochem. Sci.* **30,** 283–286 (2005).
7. Kwon, Y. T. *et al.* An essential role of N-terminal arginylation in cardiovascular development. *Science* **297,** 96–99 (2002).
8. Graciet, E., Mesiti, F. & Wellmer, F. Structure and evolutionary conservation of the plant N-end rule pathway. *Plant J.* **61,** 741–751 (2010).
9. Graciet, E. & Wellmer, F. The plant N-end rule pathway: structure and functions. *Trends Plant Sci.* **15,** 447–453 (2010).
10. Garzón, M. *et al.* PRT6/At5g02310 encodes an *Arabidopsis* ubiquitin ligase of the N-end rule pathway with arginine specificity and is not the CER3 locus. *FEBS Lett.* **581,** 3189–3196 (2007).
11. Tasaki, T. & Kwon, Y. T. The mammalian N-end rule pathway: new insights into its components and physiological roles. *Trends Biochem. Sci.* **32,** 520–528 (2007).
12. Holman, T. J. *et al.* The N-end rule pathway promotes seed germination and establishment through removal of ABA sensitivity in *Arabidopsis. Proc. Natl Acad. Sci. USA* **106,** 4549–4554 (2009).
13. Yoshida, S., Ito, M., Callis, J., Nishida, I. & Watanabe, A. A delayed leaf senescence mutant is defective in arginyl-tRNA: protein arginyltransferase, a component of the N-end rule pathway in *Arabidopsis. Plant J.* **32,** 129–137 (2002).
14. Graciet, E. *et al.* The N-end rule pathway controls multiple functions during *Arabidopsis* shoot and leaf development. *Proc. Natl Acad. Sci. USA* **106,** 13618–13623 (2009).
15. Mustroph, A. *et al.* Profiling translatomes of discrete cell populations resolves altered cellular priorities during hypoxia in *Arabidopsis. Proc. Natl Acad. Sci. USA* **106,** 18843–18848 (2009).
16. Chung, H. J. & Ferl, R. J. *Arabidopsis* alcohol dehydrogenase expression in both shoots and roots is conditioned by root growth environment. *Plant Physiol.* **121,** 429–436 (1999).
17. Nakano, T., Suzuki, K., Fujimura, T. & Shinshi, H. Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. *Plant Physiol.* **140,** 411–432 (2006).
18. Licausi, F. *et al.* HRE1 and HRE2, two hypoxia-inducible ethylene response factors, affect anaerobic responses in *Arabidopsis thaliana*. *Plant J.* **62,** 302–315 (2010).
19. Hinz, M. *et al. Arabidopsis* RAP2.2: An ethylene response transcription factor that is important for hypoxia survival. *Plant Physiol.* **153,** 757–772 (2010).
20. Papdi, C. *et al.* Functional identification of *Arabidopsis* stress regulatory genes using the controlled cDNA overexpression system. *Plant Physiol.* **147,** 528–542 (2008).
21. Hattori, Y. *et al.* The ethylene response factors SNORKEL1 and SNORKEL2 allow rice to adapt to deep water. *Nature* **460,** 1026–1030 (2009).
22. Hu, R. G. *et al.* The N-end rule pathway as a nitric oxide sensor controlling the levels of multiple regulators. *Nature* **437,** 981–986 (2005).
23. Lee, M. J. *et al.* RGS4 and RGS5 are *in vivo* substrates of the N-end rule pathway. *Proc. Natl Acad. Sci. USA* **102,** 15030–15035 (2005).
24. Takahashi, H. *et al.* A simple and high-sensitivity method for analysis of ubiquitination and polyubiquitination based on wheat cell-free protein synthesis. *BMC Plant Biol.* **9,** 39 (2009).
25. Suzuki, T. & Varshavsky, A. Degradation signals in the lysine-asparagine sequence space. *EMBO J.* **18,** 6017–6026 (1999).
26. Leonard, S. E. & Carroll, K. S. Chemical 'omics' approaches for understanding protein cysteine oxidation in biology. *Curr. Opin. Chem. Biol.* **15,** 88–102 (2011).
27. Felle, H. H. pH regulation in anoxic plants. *Ann. Bot.* **96,** 519–532 (2005).
28. Hu, R. G., Wang, H. Q., Xia, Z. X. & Varshavsky, A. The N-end rule pathway is a sensor of heme. *Proc. Natl Acad. Sci. USA* **105,** 76–81 (2008).
29. Côme, D. & Tissaoui, T. Induction d'une dormance embryonnaire secondaire chez le pommier (*Pirus malus* L.) par des atmosphères très appauvries en oxygène. *Compt. Rendus Acad. Sci.* **266,** 477–479 (1968).

**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

**Acknowledgements** M.J.H., D.J.G., S.G. and C.S.C. were supported by BBSRC grant BB/G010595/1; G.W.B. by a Marie Curie International Incoming Fellowship; N.M.I. by a MARA PhD fellowship from the Malaysian government; S.C.L., T.F. and J.B.-S. by grants NSF IOS-0750811 and NIFA 2008-35100-04528. We thank S. Liddell. Rothamsted Research receives grant-aided support from the BBSRC.

**Author Contributions** D.J.G., M.J.H., J.B.-S., F.C. and F.L.T. conceived and designed experiments. D.J.G., S.C.L., N.M.I., S.G., C.S.C., G.W.B., T.F. and F.C. performed the experiments. D.J.G., S.C.L., N.M.I., S.G., C.S.C., G.W.B., T.F., F.C. M.J.H., J.B-S. and F.L.T. analysed the data. M.J.H., D.J.G. and J.B.-S. wrote the manuscript.

**Author Information** The microarray data reported in this paper are deposited in Gene Expression Omnibus under accession number GSE29941 and are also tabulated in Supplementary Information. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to M.J.H. (michael.holdsworth@nottingham.ac.uk) or J.B.-S. (serres@ucr.edu).

## METHODS

**Growth and analysis of plant material.** *Arabidopsis thaliana* seeds were obtained from NASC, except for transgenics containing *pADH::GUS* (ref. 16) (a gift from R. Ferl). Columbia-0 (Col-0) was the wild type for all analyses. *prt6-1*, *prt6-5* and *ate1 ate2* mutants were described previously[12,14]. For the generation of transgenic *Arabidopsis* and *in vivo* protein assays, plants were grown vertically on half MS media for 7 days at 22 °C in 150 μmol $m^{-2}$ $s^{-1}$ constant light and transferred to soil after 2 weeks if required. For analysis of seedling $O_2$ deprivation survival and protein analysis, plants were grown vertically on MS medium (0.43% (w/v) MS salts, 1% (w/v) Suc and 0.4% (w/v) phytagel, pH 5.75) at 23 °C with a 16-h-day (50 μmol $m^{-2}$ $s^{-1}$) and 8-h-night cycle for 7 d. The rice (*Oryza sativa* L.) *SUB1* introgression line cv. M202(*Sub1*) was grown and submerged before cDNA isolation as described previously[3]. All plant experiments were carried out at least three times.

**Analysis of oxygen deprivation response in seeds and seedlings.** Seven-day-old *Arabidopsis* seedlings were subjected for specified durations to non-stress (NS) or hypoxia stress (HS) treatments, or subjected to hypoxia stress and returned to ambient air (re-oxygenation; R). For seedling survival, 15 Col-0 and 15 mutant seedlings were grown side by side (3 replicates). Treatments commenced at the end of the 16-h light cycle in open (NS) or sealed (HS) chambers. For HS, 99.995% argon gas was bubbled through water and into the chamber while air was expelled by positive pressure[30]. After treatment, the 15 seedlings per genotype per plate were scored as non-damaged, damaged and dead (scored 5, 3 and 1, respectively) compared to wild-type plants grown on the same plate and results analysed using the students *t*-test, as described previously[31], or seedlings were frozen under liquid nitrogen within 3 min of release before protein extraction.

Germination of *Arabidopsis* seeds (3–4 replicates of $n = 60$–100; scored on day 7 as radicle emergence) was performed at 22 °C under constant light in various oxygen tensions achieved through mixing $N_2$ and air via capillary tubes according to the apparatus described previously[29].

Wild-type plants carrying the *pADH::GUS* transgene[16] were crossed to *prt6-1* plants and homozygous *prt6-1 pADH::GUS* individuals were identified in the $F_2$ population. Seven-day-old seedlings were submerged in degassed water in the dark to induce hypoxia for the times indicated. Embryos were dissected 6 h after being imbibed. Seedlings and embryos were assayed for GUS activity and imaged following standard methods[32].

**Construction of transgenic plants and protein and RNA extractions.** To generate C-terminally HA-tagged ERF fusions of *HRE1* (At1g72360) and *HRE2* (At2g47520) driven by the 35SCaMV promoter, full-length cDNAs amplified from *Arabidopsis* total seedling cDNA were first ligated into the Entry vector pE2c and then mobilized into the Destination binary vector pB2GW7, as described previously[33]. N-terminal mutations were incorporated by changing the forward primer sequences accordingly (Supplementary Table 2). Transformation into *Agrobacterium tumefaciens* (strain GV3101 pMP90) and *Arabidopsis thaliana* was performed according to established protocols[34]. Proteins were extracted from 7-day-old homozygous $T_3$ seedlings as described[35]. Extracts were quantified using the Bio-Rad DC assay and subjected to anti-HA immunoblot analysis. For semi-quantitative RT–PCR, RNA was extracted using an RNEasy plant mini kit (Qiagen) and converted to cDNA using Superscript III Reverse transcriptase (Invitrogen). PCRs were performed with transgene-specific primers (gene-specific forward, HA-tag reverse) and *ACTIN-2* was amplified for use as a loading control (Supplementary Table 2).

***In vitro* analysis of protein stability.** To generate *Arabidopsis* and rice protein–HA fusions driven by the T7 promoter, cDNAs were PCR amplified from *Arabidopsis* total cDNA or submerged rice cDNA (M202(*Sub1*)), as described[3], and ligated into a modified version of the pTNT (Invitrogen) expression vector (pTNT3xHA). N-terminal mutations were incorporated by changing the forward primer sequences accordingly (Supplementary Table 2).

Proteins were expressed *in vitro* using the TNT T7 Coupled Reticulocyte Lysate system (Promega) according to manufacturer's guidelines, using 500 ng plasmid template. Where appropriate, 100 μM MG132 or 1 mM dipeptides (Arg-β-Ala or Ala-Ala; Sigma-Aldrich) and 150 nm Bestatin (Sigma-Aldrich) were added. Reactions were incubated at 30 °C, and samples were taken at indicated time points before mixing with protein loading dye to terminate protein synthesis. Equal amounts of each reaction were subjected to anti-HA immunoblot analysis. All blots were checked for equal loading by Ponceau staining.

**Immunoblotting.** Proteins resolved by SDS–PAGE were transferred to PVDF using a MiniTrans-Blot electrophoretic transfer cell (Bio-Rad). Membranes were probed with primary antibodies at the following titres: anti-HA (Sigma-Aldrich), 1:1,000; anti-α-tubulin (Sigma-Aldrich), 1:5,000; anti-ribosomal protein S6 (ref. 36), (1:5,000). HRP-conjugated anti-mouse secondary antibody (Santa Cruz) was used at a titre of 1:10,000. Immunoblots were developed to film using ECL western blotting substrate (Pierce).

**Alignment of MC-ERF proteins from *Arabidopsis* and rice.** Rice and *Arabidopsis* ERF proteins starting with the sequence MC were aligned and phylogenetic relationships observed using CLUSTALW[37].

**Microarray hybridization and data analyses.** Total RNA extracted from seeds or seedlings was assessed for quality using the Agilent 2100 Bioanalyser with the RNA 6000 Nano reagent kit. Biotin-labelled cRNA was synthesized using the Affymetrix 3′ IVT Express Labelling kit and hybridized against the *Arabidopsis* ATH1 genome array (GeneChip System, Affymetrix). CEL file data were processed to estimate the abundance of each expressed mRNA in two (seedling) or three (imbibed seed) biological replicate samples as described previously[15]. The microarray experiments reported here are described following MIAME guidelines and are deposited in GEO under the accession number GSE29941.

The differentially expressed genes were further analysed by use of fuzzy *k*-means clustering with the FANNY function from the Cluster package in R, as described[15]. The resulting gene-to-cluster assignments are given in Supplementary Table 1 and were visualized with the TIGR MEV program. Each gene cluster was evaluated for enrichment of specific gene functions (Gene Ontology (GO)) as described previously[38] using *Arabidopsis* gene-to-GO mappings from TAIR (http://geneontology.org; downloaded 17 May 2011).


30. Branco-Price, C., Kaiser, K. A., Jang, C. J. H., Larive, C. K. & Bailey-Serres, J. Selective mRNA translation coordinates energetic and metabolic adjustments to cellular oxygen deprivation and reoxygenation in *Arabidopsis thaliana*. *Plant J.* **56,** 743–755 (2008).
31. Mustroph, A. *et al.* Cross-kingdom comparison of transcriptomic adjustments to low-oxygen stress highlights conserved and plant-specific responses. *Plant Physiol.* **152,** 1484–1500 (2010).
32. Lucas, M. *et al.* SHORT-ROOT regulates primary, lateral, and adventitious root development in *Arabidopsis*. *Plant Physiol.* **155,** 384–398 (2011).
33. Dubin, M. J., Bowler, C. & Benvenuto, G. A modified Gateway cloning strategy for overexpressing tagged proteins in plants. *Plant Methods* **4,** 3 (2008).
34. Swarup, R. *et al.* Root gravitropism requires lateral root cap and epidermal cells for transport and response to a mobile auxin signal. *Nature Cell Biol.* **7,** 1057–1065 (2005).
35. Martinez-Garcia, J. F., Monte, E. & Quail, P. H. A simple, rapid and quantitative method for preparing *Arabidopsis* protein extracts for immunoblot analysis. *Plant J.* **20,** 251–257 (1999).
36. Williams, A. J., Werner-Fraczek, J., Chang, I. F. & Bailey-Serres, J. Regulated phosphorylation of 40S ribosomal protein S6 in root tips of maize. *Plant Physiol.* **132,** 2086–2097 (2003).
37. Thompson, J. D., Higgins, D. G. & Gibson, T. J. Clustal-W - Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22,** 4673–4680 (1994).
38. Horan, K. *et al.* Annotating genes of known and unknown function by large-scale coexpression analysis. *Plant Physiol.* **147,** 41–57 (2008).