LETTER

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Oxygen sensing in plants is mediated by an N-end rule pathway for protein destabilization

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The majority of eukaryotic organisms rely on molecular oxygen for respiratory energy production[1]. When the supply of oxygen is compromised, a variety of acclimation responses are activated to reduce the detrimental effects of energy depletion[2–4]. Various oxygen-sensing mechanisms have been described that are thought to trigger these responses[5–9], but they each seem to be kingdom specific and no sensing mechanism has been identified in plants until now. Here we show that one branch of the ubiquitin-dependent N-end rule pathway for protein degradation, which is active in both mammals and plants[10,11], functions as an oxygen-sensing mechanism in *Arabidopsis thaliana*. We identified a conserved amino-terminal amino acid sequence of the ethylene response factor (ERF)-transcription factor RAP2.12 to be dedicated to an oxygen-dependent sequence of post-translational modifications, which ultimately lead to degradation of RAP2.12 under aerobic conditions. When the oxygen concentration is low—as during flooding—RAP2.12 is released from the plasma membrane and accumulates in the nucleus to activate gene expression for hypoxia acclimation. Our discovery of an oxygen-sensing mechanism opens up new possibilities for improving flooding tolerance in crops.

Tolerance to submergence and low oxygen availability (hypoxia) have been considered to be influenced by different members of subgroup VII of the ERF transcription factor family in *Arabidopsis* (RAP2.12 (ref. 12), RAP2.2 (ref. 13); HRE1 and HRE2 (ref. 14)) and rice (SUB1 (ref. 15), SK1 and SK2 (ref. 16)). Here, we reveal the mechanism by which molecular oxygen acts upon RAP2.12 (At1g53910) to trigger molecular acclimation responses. RAP2.12 is highly homologous to RAP2.2 and is widely conserved in higher plants (Supplementary Fig. 1). It is constitutively expressed throughout the entire plant (Supplementary Fig. 2) and further upregulated in leaves upon hypoxia, but not by the ethylene precursor 1-aminocyclopropane-1-carboxylic-acid (ACC) (Supplementary Fig. 3). RAP2.12 positively regulates gene transcription *in planta* via a conserved carboxy-terminal motif (Supplementary Fig. 4). Constitutive overexpression of RAP2.12 (*35S::RAP2.12*) did not significantly affect the phenotype of *Arabidopsis* plants when grown aerobically (Fig. 1a, b). However, submergence tolerance of independently transformed *35S::RAP2.12* plants increased with respect to the wild-type control, as demonstrated by the increased number and dry weight of plants that recovered from submergence (Fig. 1a, c, d), which can be explained by the faster and stronger induction of hypoxia-responsive genes during the flooding treatment in *35S::RAP2.12* plants (Supplementary Fig. 5). Interestingly, different flooding-tolerance strategies in two wild *Rumex* species correlated with the differential induction of *ERF1*, which is the orthologue of *RAP2.12* (Supplementary Fig. 6). In contrast, constitutive expression of RAP2.12 with a haemagglutinin (HA)-peptide tag at its N terminus (*35S::HA::RAP2.12*) resulted in a reduction of plant growth in air (Fig. 1a, b). Concomitantly, tolerance to submergence decreased as compared to the wild type (Fig. 1c). Similar results were observed when a version of RAP2.12 was expressed from which the first 13 amino acid residues were deleted (*35S::Δ13RAP2.12*). It thus seemed that manipulating the N-terminal amino acid sequence obstructed the regulative function of RAP2.12 already under aerobic conditions, thereby reducing the vigour and stress tolerance of the plants.

To understand the impact of the N-terminal modifications on the activity of RAP2.12, we investigated which genes are expressed under the control of RAP2.12. We found that under aerobic conditions

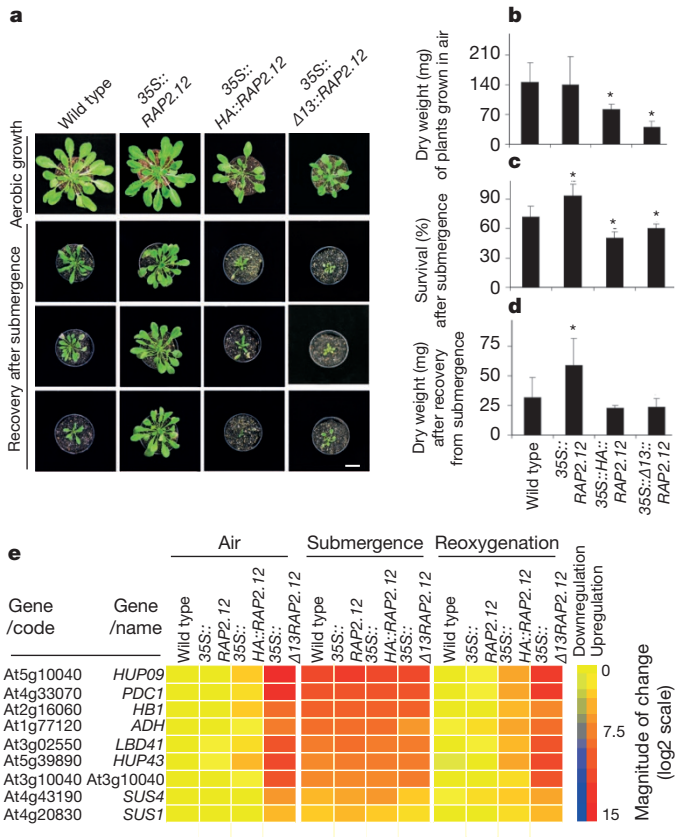

Figure 1 | The transcription factor RAP2.12 regulates hypoxia tolerance of plants. **a**, The effect of overexpression of RAP2.12, HA::RAP2.12 or Δ13RAP2.12 on plant growth in air, or after submergence. Scale bar, 2 cm. **b**, Dry weight of 7-week-old rosette leaves from air-grown plants ($n = 20$). **c**, Percentage of plants surviving flooding-induced hypoxia ($n = 4$). **d**, Dry weight of rosette leaves from surviving plants, 2 weeks after the flooding treatment ($n = 20$). **e**, Differential expression of hypoxia-responsive genes (reference: wild type in air). Numeric expression values are shown in Supplementary Table 1. Data are presented as mean ± s.d. $^*P < 0.05$, one-way ANOVA.

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35S::RAP2.12 plants exhibited a slight increase in the expression of hypoxia marker genes, whereas during flooding the expression of these hypoxia marker genes was more strongly upregulated in plants overexpressing RAP2.12 as compared to wild-type plants during flooding (Fig. 1e and Supplementary Table 1). During re-oxygenation, the expression of the hypoxia marker genes was rapidly downregulated in both wild-type plants and *35S::RAP2.12*, whereas in *35S::HA::RAP2.12* and *35S::Δ13RAP2.12* the level of expression remained high, as before the flooding treatment. The correlation between this expression pattern of hypoxia response genes and the reduced growth and recovery after flooding that is observed for plants overexpressing *HA::RAP2.12* and *Δ13RAP2.12* indicates that proper upregulation of the hypoxia response genes during flooding as well as downregulation of these genes during recovery from flooding are both required for optimal plant acclimation. The activation of hypoxic gene expression by RAP2.12 was further confirmed by the observation that RAP2.12 induced a luciferase (*Luc*) reporter gene when its promoter contained the motif ATCTA (Supplementary Fig. 7), which was previously identified as a hypoxia-responsive element in plants[17]. On the other hand, the relatively small effect of *35S::RAP2.12* on gene expression under aerobic conditions (Fig. 1e) indicated that an additional regulatory mechanism reliant on sensing of low oxygen concentrations is needed to induce hypoxic gene expression. Interestingly, this requirement was abolished when the N terminus of RAP2.12 was modified either by fusing the HA-peptide tag to the protein (*35S::HA::RAP2.12* in Fig. 1e), or by deleting its first conserved amino acid residues (*35S::Δ13RAP2.12* in Fig. 1e and Supplementary Fig. 8), indicating that the N terminus of RAP2.12 has an important role in the regulation of the oxygen-dependent activation of the transcription factor.

Further comparative analysis of a full-genome expression profile of the hypoxic response in wild-type plants and the differential regulation of genes by expressing the *HA::RAP2.12* construct under aerobic conditions revealed that the genes that were most strongly up- or downregulated by HA::RAP2.12 were also differentially expressed under hypoxia (Supplementary Fig. 9 and Supplementary Table 2). Similarly, the silencing of *RAP2.12* and its closest homologue *RAP2.2* using an artificial microRNA approach reduced the induction of hypoxic gene expression by low oxygen (Supplementary Fig. 10 and Supplementary Tables 3 and 4). Given that the messenger RNA stability of *RAP2.12* was not affected by the additional nucleotides encoding the N-terminal peptide tag (Supplementary Fig. 11), we concluded that post-translational modifications of the N-terminal amino acid residues of RAP2.12 are involved in regulating the activity of this transcription factor, which is required to induce hypoxia core-response genes.

We further investigated the role of the N-terminal amino acid residues by determining the subcellular localization of RAP2.12 fused to green fluorescent protein (GFP). Under aerobic conditions, the fusion protein localized to the plasma membrane; however, upon hypoxia it accumulated in the nucleus (Fig. 2a and Supplementary Fig. 12). Remarkably, upon re-oxygenation the RAP2.12::GFP signal fully disappeared within 1 h (Fig. 2a). After deleting the conserved N-terminal amino acid residues of RAP2.12 (*35S::Δ13RAP2.12::GFP*), the transcription factor was observed in both the cell membrane and the nucleus under aerobic conditions (Fig. 2a). However, under hypoxia, the

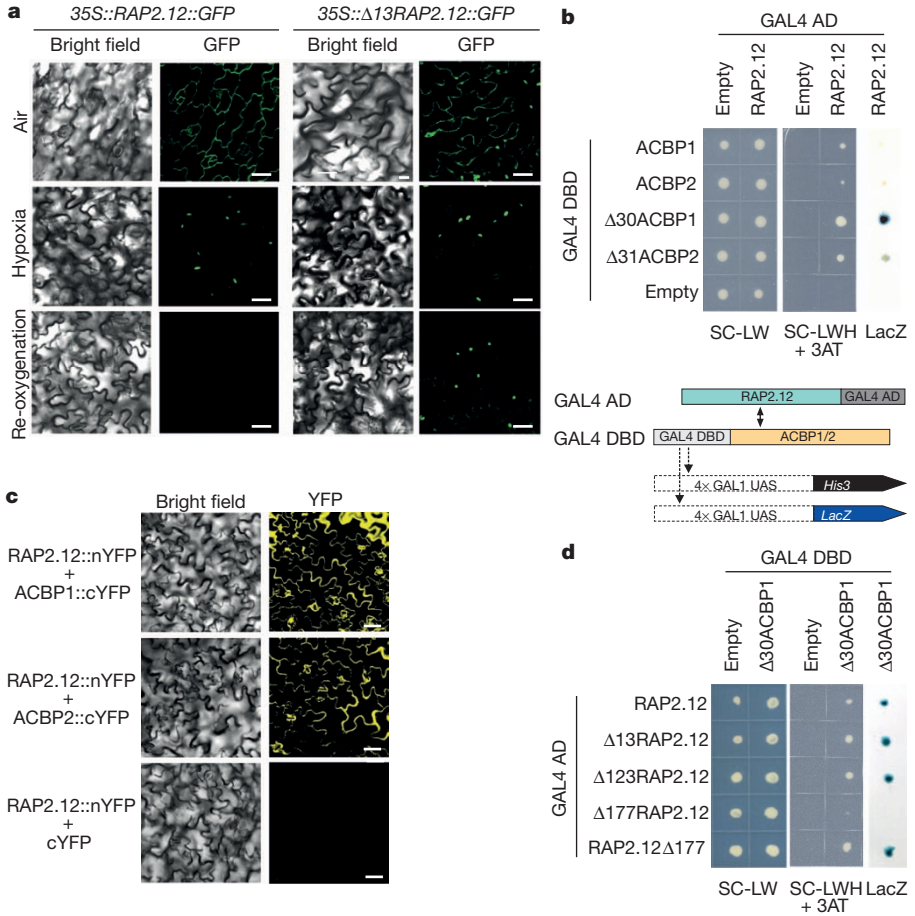

Figure 2 | RAP2.12 is membrane localized and re-localizes in the nucleus upon hypoxia. **a**, Subcellular localization of stably transformed GFP-fused RAP2.12 and Δ13RAP2.12. Localization controls are shown in Supplementary Fig. 12. **b**, Yeast two-hybrid analysis showing interaction between RAP2.12 and ACBP1 and ACBP2. **c**, Bimolecular fluorescence complementation of YFP confirming interaction between RAP2.12 and ACBP1 and ACBP2. **d**, Yeast two-hybrid analysis between various truncated RAP2.12 proteins and Δ30ACBP1. Names of genes are explained in a pictogram shown in Supplementary Fig. 16. AD, activation domain; DBD, DNA-binding domain; UAS, upstream activator sequence. Scale bars, 10 μm.

membrane association disappeared, the protein accumulated only in the nucleus and remained there even after re-oxygenation (*35S::Δ13RAP2.12::GFP* in Fig. 2a). Thus, manipulation of the conserved N terminus of RAP2.12 seems to affect the oxygen-dependent subcellular localization of the transcription factor and, moreover, stabilizes the protein under aerobic conditions.

As RAP2.12 has no hydrophobic domains that could explain its localization at the plasma membrane, we searched for interaction partners of the transcription factor. Yeast two-hybrid analyses (Fig. 2b) and bimolecular fluorescence complementation (BiFC) analysis (Fig. 2c) revealed an interaction between RAP2.12 and the membrane-localized acyl-CoA-binding proteins ACBP1 and ACBP2 (ref. 18), as had been shown previously for ACBP2 and RAP2.3 (ref. 19). The interaction between RAP2.12 and ACBP depended on an amino acid sequence between position 123 and 177, which covers the RAYD motif, a sequence already known to mediate protein–protein interactions[20] (Fig. 2d).

The essential role of the N-terminal residues of RAP2.12 is further supported by the conservation of the first amino acids in almost all members of ERF subfamily VII (Supplementary Fig. 8). The specific sequence of their conserved N terminus qualifies ERF-VII proteins as candidate substrates of the N-end rule pathway[21,22] (Fig. 3a). According to this pathway the terminal Met is removed from the protein by methionine aminopeptidase (MetAP) when the second amino acid of the protein is Cys[23] (Supplementary Fig. 13 and Supplementary Table 5). Terminal Cys is oxidized to cysteine sulphenic acid in an oxygen-dependent manner before arginine transferase (ATE) conjugates an Arg residue to the protein[10,11]. This triggers subsequent ubiquitination by the ligase PROTEOLYSIS 6 (PRT6)[24] and targets the protein to the proteasome for degradation[25], which can occur in both the cytosol and the nucleus[26]. Transient expression of RAP2.12::GFP in *ate1ate2* or *prt6* knockout plants resulted in accumulation of the transcription factor in the nucleus both during aerobic and hypoxic conditions as well as after re-oxygenation (Fig. 3b), similar to what we observed by deleting the N-terminal sequence (Fig. 2a) or after incubation with the proteasome inhibitor MG132 (Supplementary Fig. 14). Western blot analyses showed that the amount of RAP2.12 increased under hypoxia and decreased again after re-oxygenation in the wild type but not in *ate1ate2* or *prt6* (Fig. 3c). The tolerance to submergence of *ate1ate2* and *prt6* rosette plants was reduced (Supplementary Fig. 15), in line with the negative impact of *35S::HA::RAP2.12* and *35S::Δ13RAP2.12* on survival (Fig. 1a, b). Lastly, exchanging the N-terminal Cys with Ala (*35S::MAG-RAP2.12::GFP*) resulted in a GFP signal in the nucleus, similar to what we observed in any of the

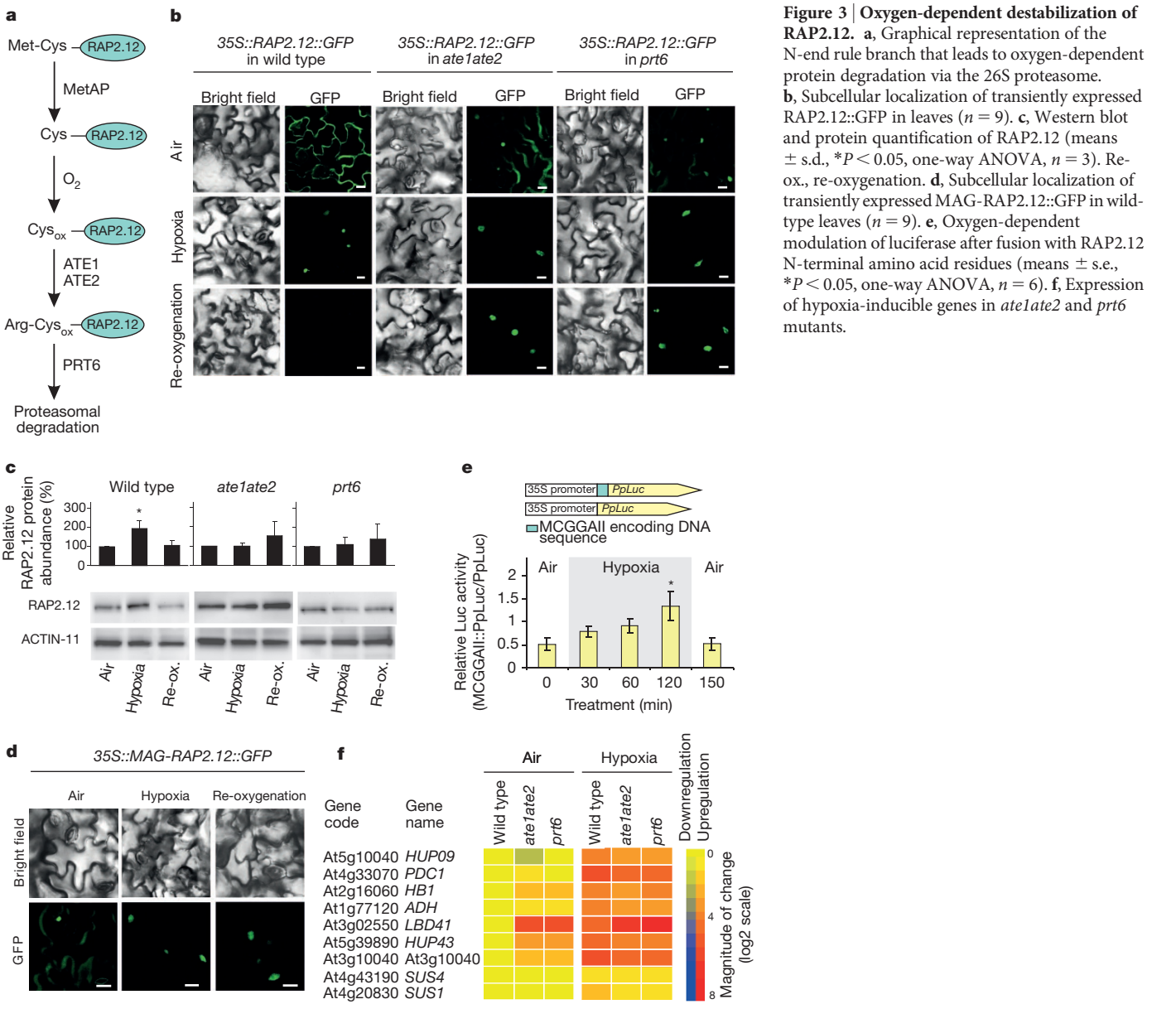

Figure 3 | Oxygen-dependent destabilization of RAP2.12. **a**, Graphical representation of the N-end rule branch that leads to oxygen-dependent protein degradation via the 26S proteasome. **b**, Subcellular localization of transiently expressed RAP2.12::GFP in leaves ($n = 9$). **c**, Western blot and protein quantification of RAP2.12 (means ± s.d., $*P < 0.05$, one-way ANOVA, $n = 3$). Re-ox., re-oxygenation. **d**, Subcellular localization of transiently expressed MAG-RAP2.12::GFP in wild-type leaves ($n = 9$). **e**, Oxygen-dependent modulation of luciferase after fusion with RAP2.12 N-terminal amino acid residues (means ± s.e., $*P < 0.05$, one-way ANOVA, $n = 6$). **f**, Expression of hypoxia-inducible genes in *ate1ate2* and *prt6* mutants.

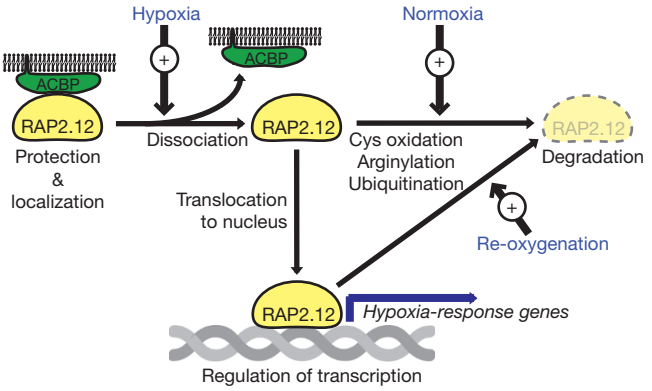

Figure 4 | Model describing the oxygen sensor mechanism in plants. The transcription factor *RAP2.12* is constitutively expressed under aerobic conditions. RAP2.12 protein is always present, bound to ACBP to prevent RAP2.12 from moving into the nucleus under aerobic conditions and to protect it against proteasomal degradation in air. Upon hypoxia, RAP2.12 moves into the nucleus, where it activates anaerobic-gene expression. Upon re-oxygenation, RAP2.12 is rapidly degraded via the N-end rule pathway and proteasome-mediated proteolysis to downregulate the hypoxic response.

other approaches to modify the N-end rule pathway (Fig. 3d). All this indicates that the lifetime of RAP2.12 is controlled by the N-end rule pathway for proteasomal protein degradation.

Next, we investigated whether an oxygen-dependent N-end rule pathway is active in plants and whether it regulates the oxygen-dependent activation of hypoxic gene expression. Fusion of the first conserved N-terminal amino acid residues from RAP2.12 to the Luc reporter protein resulted in an increase of the normalized Luc activity under hypoxic conditions and reduced Luc activity upon re-oxygenation, as predicted by the Cys-oxidation-dependent branch of the N-end rule pathway (Fig. 3e). In addition, constitutive upregulation of hypoxia marker genes was observed under aerobic conditions in plants with reduced ATE and PRT activities (Fig. 3f). This indicates that the oxygen-dependent oxidation of the terminal Cys of RAP2.12 prevents hypoxic gene expression via the destabilization of RAP2.12 in air. Only when the oxygen concentration decreases is Cys oxidation prevented, and the now stably accumulating RAP2.12 can induce the expression of genes involved in the hypoxic response (Fig. 4). Here, we have shown that this oxygen-dependent Cys oxidation is adopted by the ERF-VII factor RAP2.12 and—together with its oxygen-dependent re-localization—triggers the hypoxia-acclimation response in *Arabidopsis*.

METHODS SUMMARY

Unless specifically indicated in the text, low oxygen (hypoxia) conditions used in this study were always maintained at 1% (v/v) oxygen. Full details of the materials and experimental procedures are provided in Methods.

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

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

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Author Contributions F.L., M.K., D.A.W. and B.G. performed the experiments. F.M.G. carried out the bioinformatical analysis. F.L., L.A.C.J.V., P.P. and J.T.v.D. designed the experiments. F.L., P.P. and J.T.v.D. wrote the manuscript. All the authors discussed and commented on the content of the paper.

Author Information The raw data files of the microarray experiments have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo; accession number: GSE29187). The gene sequences for the *Rumex spp.* used in this work have been deposited at NCBI (*RaERF1*: JF968115; *RaERF2*: JF968116; *RpERF1*: JF968117; *RpERF2*: JF968118; and *RpERF3*: JF968119). 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 F.L. (f.licausi@sssup.it) or J.T.v.D. (dongen@mpimp-golm.mpg.de).

METHODS

Plant materials. *A. thaliana* Columbia-0 (Col-0) was used as wild-type ecotype, as described in the figure legends. Double *ate1ate2* knockout seeds were provided by E. Graciet, *prt6* knockout seeds (line EOL4) were obtained from the Institute of Agronomic Research, *35S::GFP* seeds were provided by M. Kawai-Yamada.

Growth conditions and phenotypic evaluation. Seeds were sown in moist soil, stratified at 4 °C in the dark for 48 h and germinated at 22 °C day/18 °C night with a photoperiod of 8 h light and 16 h darkness. For all experiments 5-week-old plants were used. Low oxygen (1% (v/v) oxygen in air) treatments were performed as described previously[17]. Flooding tolerance was assayed using three independent transgenic lines. Plants were submerged with deionized water in 15-cm-high plastic boxes and kept in the dark. Leaves were at 5 cm under the water surface. After 84 h, the water was removed from the boxes and photoperiodic conditions (8 h/16 h, light/dark) were restored. Tolerance assays were repeated four times by using 10–20 plants per genotype each time. *Rumex spp.* cultivation and submergence treatment were performed as described previously[27].

Cloning of the various constructs. Coding sequences (CDSs) were amplified from a cDNA template using Phusion High Fidelity DNA-polymerase (New England Biolabs). An artificial microRNA (amiRNA) against RAP2.12 was generated by overlapping PCR using the pRS300 vector as backbone. All open reading frames were cloned into pENTR/D-TOPO (Invitrogen). The resulting entry vectors were recombined into destination vectors using the LR reaction mix II (Invitrogen) to obtain the expression vectors. A complete list of all destination vectors and primers used is provided in Supplementary Tables 6 and 7, respectively.

Plant transformation. Stable transgenic plants were obtained using the floral dip method[28]. T0 seeds were screened for kanamycin or phosphinotricine resistance and single-insertion lines were identified as described previously[13] Transient leaf transformations using 3-week-old plants were performed as described previously[29]. All transient expression essays were repeated at least three times using independently grown plants. Each time the experiment was repeated, we transformed leaves from three independent plants. So, at least nine independent transformations from at least three different plant cultures were analysed.

qRT–PCR. RNA extraction, removal of genomic DNA, cDNA synthesis and qRT–PCR analyses were performed as described previously[13]. For *35S::RAP2.12*, *35S::HA::RAP2.12*, *amiRAP2.2-12* and *35S::Δ13RAP2.12* three independent transgenic lines were used and the average expression value was calculated. For all the other genotypes, three independent biological replicates were used.

Microarrays. Three independent *RAP2.12* overexpressors or *RAP2.2-RAP2.12* silenced lines were grown in soil for 5 weeks and then subjected to a treatment with 1% oxygen in the dark for 90 min. Total RNA from whole rosettes was extracted as described for the qRT–PCR analyses. Hybridization and scanning procedures were performed by NASC (http://arabidopsis.info/). Microarray analysis and data quality control were performed as described previously[13] using Robin[30]. Normalization of the raw data and an estimation of signal intensities were carried out using the Genechip Robust Multiarray Average (GC-RMA) methodology[31]. Differential gene expression analysis was carried out using limma[32], with a Benjamini–Hochberg *P*-value correction[33]. Microarray data sets were deposited in a public repository with open access (accession number GSE29187; http://www.ncbi.nlm.nih.gov/geo/).

Confocal imaging. For GFP and YFP imaging, leaves from independent stable or transiently transformed 4-week-old plants were analysed with a Leica DM6000B/SP5 confocal microscope (Leica Microsystems).

Reporter transactivation assay. *Arabidopsis* mesophyll protoplasts were used to identify the region responsible for the trans-activation activity of RAP2.12. The DNA-binding domain from *Saccharomyces cerevisiae* was fused at the N terminus of RAP2.12 and its deletion variants. The UAS fused to a minimal 35S promoter was inserted into pGreenII-800LUC to generate a reporter and normalization vector. Non-recombined pDBD-GW vector was used as a negative control. Protoplasts were prepared according to a previously described method[34] and transfected using 5 μg plasmid DNA each. A dual luciferase reporter assay was performed as described previously[16].

Protein stability assay using the Luc reporter system. Leaves of *A. thaliana* Col-0 were transformed with either a *35S::PpLuc* or a *35S::MCGGAII::PpLuc* constructs (both containing also a *35S::RrLuc* cassette for normalization purposes). Normalized luciferase activity (PpLuc/RrLuc) was measured as described previously[17] and Luc protein stability was evaluated as the ratio between *MCGGAII::PpLuc* and *PpLuc* transfected leaves. The experiment was repeated three times using five independent replicates in each repetition.

Yeast two-hybrid assay. The ProQuestTm Two-hybrid System (Invitrogen) was used. PExpTM32/Krev1 and pEXPTM22/RalGDS-wt were used as positive controls, and pDESTTM32 and pDESTTM22 as negative controls. *S. cerevisiae* strain Mav203 was transformed with the different combinations of bait, prey and control vectors (Supplementary Fig. 16). Colonies containing both vectors were selected by plating at 28 °C to select colonies containing an interacting protein partner for 3 days on minimal selective dropout medium lacking Leu and Trp (SC-LW medium). They were subsequently replicated on selective dropout medium (SC-LWH+3AT medium) lacking Leu, Trp, His and supplemented with 10 mM 3-aminotriazole (3AT). The strength of the interaction was further verified by β-galactosidase staining (LacZ) following the manufacturer's instructions.

SC-LW, control medium without Leu and Trp; SC-LWH+3AT, selective medium without Leu, Trp, His and with 3AT.

BiFC. *In planta* protein interactions were investigated with bimolecular fluorescence complementation in an *Arabidopsis* transient expression system as described previously[35].

SDS–PAGE and western blotting. Protein samples from total tissue extracts were separated by SDS–PAGE on 10% acrylamide midigels (Biorad) and then transferred onto a polyvinylidene difluoride membrane (BioRad). Incubations with the antiserum and the secondary antibody conjugated to horseradish peroxidase (Agrisera) were performed following the method recommended for the ECL Plus western blotting detection system (GE Healthcare).

Polyclonal anti-RAP2.12 antibodies were affinity purified at Genscript laboratories after being raised in rabbits against a RAP2.12/RAP2.2 specific synthetic peptide (NLKGSKKSSKNRSN). Lyophilized antibody was re-suspended to an approximate concentration of 1 $\mu g\,ml^{-1}$. A monoclonal antibody against *Arabidopsis* ACTIN-11 (Agrisera, AS10 702) was used to confirm equal loading and transfer. Densitometric analysis of the protein signals on the western blots was performed with the software package UVP VisionWorks LS (Ultra-Violet Products). Normalization was carried out using the ACTIN-11 signal and setting to 100 the relative protein signal value for each of the 'air' controls.

Statistical analyses. Significant variations between genotypes or treatments were evaluated statistically by Sigmaplot using either a *t*-test, one-way or two-way ANOVA where appropriate. Mean values that were significantly different ($P < 0.05$) from the control or wild-type treatment are marked with an asterisk. The statistical evaluation of the microarray experiments is described earlier.

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