An apical hypoxic niche sets the pace over shoot meristem activity

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Abstract

Complex multicellular organisms, such as higher plants and animals, evolved on Earth in an oxygen rich atmosphere[1]. Their tissues, including stem cell niches, require continuous oxygen provision for efficient energy metabolism[2]. Remarkably, maintenance of the pluripotent state of animal stem cells requires hypoxic conditions, whereas higher oxygen tension promotes cell differentiation[3]. Using a combination of genetic reporters and *in vivo* oxygen measurements, we demonstrate that the plant shoot meristems develop embedded in a low oxygen niche and such hypoxic conditions are required to regulate the production of new leaves. We show that shoot meristem-localised hypoxia inhibits the proteolysis of a novel N-

25 degron pathway[4,5] substrate, LITTLE ZIPPER 2 (ZPR2), which evolved to control the activity
26 of Class III homeodomain leucine zipper (HD-ZIP III) transcription factors[6–8] and thereby
27 regulates shoot meristem activity. Our results reveal oxygen as a diffusible signal involved in
28 the control of stem cell activity in plants grown under aerobic conditions, suggesting that
29 spatially distinct oxygen distribution affects plant development. Molecularly, this signal is
30 translated into transcriptional regulation by an unexplored role of the N-degron pathway
31 linking the regulation of metabolic activity and development in plants.

32 **Main text**

33 The shoot apical meristem (SAM) of plants, responsible for the production of aboveground
34 organs, is characterised by a multi-layered structure where a central zone is dedicated to the
35 maintenance of stem cell identity, while a peripheral region gives rise to whorls of new
36 organs[9]. Spatial coordination of primordia production, known as phyllotaxis, is coordinated
37 by polar auxin transport[10], whereas the regulation of the temporal pattern of such process,
38 termed 'the plastochrone', is less well understood[11,12]. Studies in a variety of plant species
39 have identified a number of genes involved in plastochrone length that constitute a complex
40 signalling network downstream of exogenous and endogenous cues[13–17]. Among these stimuli,
41 oxygen is a highly reactive, diffusible molecule and retains a remarkable potential to attune
42 developmental patterns according to the metabolic needs. However, the role of oxygen levels
43 in the SAM has not been assessed so far, since this tissue consists of a small group of densely
44 packed cells surrounded by layers of developing primordia.

45 To elucidate this aspect, we exploited a micro-scale Clark type oxygen electrode whose
46 miniaturised probe is sufficiently thin to measure oxygen levels at micrometric resolution
47 (Extended Data Fig. 1a-b). Measurements through the shoot apex in the longitudinal and
48 radial directions showed a sharp decline in the oxygen concentration in a region broadly

49 overlapping with the stem cell niche (Fig. 1a and Extended Data Fig. 1), thus revealing the
50 existence of a hypoxic niche enclosing the SAM. In agreement with these observations, the
51 expression of 55% of the core hypoxia-inducible genes[18] was significantly higher in the SAM
52 as compared to juvenile leaves in public transcriptome databases (Extended Data Fig. 2).
53 Moreover, a synthetic promoter based on the Hypoxia Responsive Promoter Element
54 (HRPE)[19,20] drove β-glucuronidase (GUS) activity in SAM and leaf primordia in normoxia
55 (21% O_2), whereas reporter staining progressively spread to outer tissues in plants exposed to
56 increasing hypoxic conditions (10 and 5% O_2) (Extended Data Fig. 3a-c). *pHRPE* activity, as
57 well as that of endogenous hypoxia-responsive promoters, was maintained in reproductive
58 meristems (Fig. 1b, Extended Data Fig. 4). Conversely, a 12 h hyperoxic (80% O_2) treatment
59 strongly decreased GUS activity in the shoot apex and repressed hypoxia-inducible genes in
60 SAM-enriched samples (Extended Data 3a,c,d), confirming the perception of hypoxic
61 conditions by this tissue. We also measured hypoxia and high level of low oxygen-inducible
62 transcripts in the SAM of *Solanum lycopersicum* var. Micro-Tom, indicating that O_2 gradients
63 are a common feature in shoot apices (Extended Data Fig. 5). Remarkably, the low
64 availability of oxygen in the SAM did not appear to be a limiting factor for the production of
65 new organs. In fact, inhibition of the hypoxic response in shoot meristems by exposing
66 seedlings to hyperoxic (80% O_2) conditions did not improve, but rather impaired shoot
67 meristem activity (Fig. 1c,d). This result highlights the importance of maintaining the SAM
68 embedded in a low oxygen niche for functional purposes.
69
70 To elucidate how the establishment of a hypoxic niche regulates SAM activity, we searched
71 for oxygen-dependent molecular switches possibly involved in developmental processes.
72 Among SAM related proteins, we identified LITTLE ZIPPER 2 (ZPR2) as a possible
73 proteolytic target of the oxygen-dependent branch of the N-degron pathway [21–23] (Fig. 2a-b).
74 According to this pathway, a penultimate N-terminal Cys residue (Cys2) is exposed at the N-

75 terminus by removal of the initial methionine by a Met aminopeptidase[24]. In the presence of
76 nitric oxide and oxygen, Cys can be oxidized by Plant Cysteine Oxidases (PCOs)[25,26] and
77 subsequently arginylated by Arg-aminotransferases (ATEs)[27,28]. Finally, N-terminally Arg-
78 labelled proteins can be polyubiquitinated by the single subunit E3 ligase PROTEOLYSIS 6
79 (PRT6) and thereby targeted to proteasomal degradation[29] (Fig. 2a).

80 We focused on ZPR2 since it belongs to a protein family involved in organ polarity and shoot
81 apical meristem activity[7,8]. In flowering plants, the ZPR family is comprised of two separate
82 clades, one of which is distinguishable by a conserved N-terminal MC-motif (Fig. 2b,
83 Extended Data Fig. 6). We first tested whether ZPR2 is a true target of the N-degron pathway,
84 combining confocal microscopy, biochemical assays and immuno-blots. A ZPR2-GFP fusion
85 protein only accumulated following hypoxia treatment, whereas addition of an alanine
86 preceding the N-terminal cysteine led to stabilization under aerobic conditions (Fig. 2c-d).
87 Surprisingly, hypoxia caused a decrease in MAC-ZPR2-GFP abundance, either due to
88 repression of translation or by proteolytic counterbalance, since the relative mRNA levels
89 were not affected (Fig. 2d and Extended Data Fig. 7a). Moreover, the expression of the
90 endogenous (*MC)ZPR2-GFP* in a *prt6* mutant background strongly promoted protein stability
91 (Fig. 2c-d). Together with additional tests by alternative ZPR2-GUS and ZPR2-PpLUC
92 protein fusions, these results supported the requirement of Cys2 for ZPR2 degradation in the
93 presence of oxygen (Fig. 2c-d, Extended Data Fig. 7b-c). We therefore hypothesized that
94 ZPR2 could behave as a *bona fide* N-degron pathway substrate in its own expression domain.

95 To investigate the expression pattern of *ZPR2*, we fused the *ZPR2* promoter to a chimeric
96 *GFP-GUS* reporter and observed that this gene was specifically active in the organizing centre
97 of the SAM during vegetative and reproductive development (Fig. 3a), which occupies a
98 hypoxic niche as previously shown (Fig. 1 and Extended Data Fig. 1 and 4). Thus, we
99 assessed ZPR2 stability under the naturally occurring oxygen gradient present in the SAM,

100 using a *pZPR2:ZPR2-GUS* construct. A moderate GUS signal was observed in the shoot apex,
101 suggesting that this protein is locally stable during normal development (Fig. 3b). As
102 expected, application of hyperoxia decreased ZPR2-GUS stability while hypoxia promoted it
103 (Fig. 3b, Extended Data Fig. 7d). This indicates that SAM-localized hypoxia constitutes a
104 post-transcriptional checkpoint for ZPR2 protein accumulation in the stem cell niche.

105 To examine the role played by ZPR2 in the SAM, we exploited two T-DNA insertion mutants
106 with abolished *ZPR2* expression (Extended Data Fig. 8a-d). Both *zpr2-2* and *zpr2-3* exhibited
107 a decreased leaf initiation rate (leaves d^{-1}) as compared to a Col-0 wild type (Fig. 3c-d and
108 Extended Data Fig. 8e). This phenotype was partially complemented by introducing a
109 *pZPR2:ZPR2-FLAG* construct in the *zpr2-3* background (Extended Data Fig. 8f-g), indicating
110 that ZPR2 is required to sustain the leaf induction rate by SAM cells.

111 At the mechanistic level, ZPR proteins have previously been shown to act as negative
112 regulators of class III homeodomain-leucine zipper (HD-ZIP III), a group of transcription
113 factors extensively characterised as essential for SAM activity[6,30,31]. To investigate which
114 HD-ZIP III are controlled by ZPR2, we analysed the effect of this little zipper protein on the
115 transactivation capacity of each HD-ZIP III members using a transient transformation system
116 coupled to a dual luciferase assay. In mesophyll protoplasts, the overexpression of ZPR2
117 repressed activity of REVOLUTA (REV), PHABULOSA (PHB) and HOMEOBOX GENE 8
118 (ATHB8) on the *ZPR1* promoter (Fig. 4a). Remarkably, PHAVOLUTA (PHV) did not
119 activate *pZPR1* while CORONA (CNA) activity was not significantly affected by ZPR2 (Fig.
120 4a). C-terminal tagging with GUS inhibited the repressive effect of ZPR2 without affecting its
121 N-degron pathway susceptibility (Extended Data Fig. 7b, 9a and Fig. 3b). Using bimolecular
122 fluorescence complementation (BiFC), we also confirmed the interaction between ZPR2 and
123 REV, ATHB8 and PHB *in vivo* (Fig. 4b). These results suggest that ZPR2 represses the
124 activity of specific HD-ZIP III members that act as regulators of SAM development.

125 To elucidate which genes act downstream of the HD-ZIP III-ZPR2 regulatory module, we
126 generated estradiol inducible *ZPR2* overexpressor lines and analysed the mRNA levels of
127 known HD-ZIP III targets[32]. Induction of *ZPR2* under hypoxia, but not under aerobic
128 conditions, led to repression of REV target genes (Fig. 4c). Additionally, we visualized the
129 repression imposed by ZPR2 in the shoot apex by means of GUS staining in promoter-
130 reporter lines generated for *HECATE1* (*HEC1*), *PHYTOSULFOKINE 5 PRECURSOR* (*PSK5*)
131 and *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1)* (Extended Data
132 Fig. 9). Interestingly, some of the genes affected by *ZPR2* have been previously shown to
133 alter *WUSCHEL* expression, and play a role in SAM activity[33–35]. To confirm that ZPR2 can
134 also alter the expression of these genes in its endogenous SAM expression domain, we
135 analysed the expression pattern of HD-ZIP III target genes in the *zpr2-3* loss-of-function
136 mutant. Several HD-ZIP III targets expressed to a higher extent in *zpr2-3*, showing that ZPR2
137 is indeed required to repress these genes (Extended Data Fig. 10a-b). Finally, we observed
138 that ectopic *ZPR2* expression occasionally caused meristem arrest, whose frequency was
139 significantly increased under ZPR2-stabilising hypoxic conditions (2% O_2) (Extended Data
140 Fig. 8h-i). Intriguingly, this phenotype resembles that of plants characterized by severely
141 reduced HD-ZIP III activity[6,36,37]. These results revealed a regulatory system in which HD-
142 ZIP IIIs are modulated by ZPR2 that, in turn, requires hypoxia to be stabilized at its specific
143 site of expression (Extended Data Fig. 10c).

144 Together with recent reports on underground seedling development[38], vernalization[39] and
145 seasonal burst of grape buds[40], our study supports the involvement of a Cys/Arg branch of the
146 N-degron pathway to control plant development in an oxygen-dependent manner. However,
147 here hypoxic conditions were not studied as a limiting factor but rather as an established
148 condition in SAM cells to promote leaf organogenesis. Therefore, oxygen assumes a new role
149 as an endogenous diffusible molecule with signalling functions connecting developmental

150 processes to metabolic activity. In an evolutionary perspective, stem cells evolved
151 independently in plants and animals converging to being embedded in a hypoxic niche[3],
152 where the oxygen gradients act as a regulatory cue. Therefore, precise manipulation of oxygen
153 provision or modulation of its sensory mechanisms retains remarkable potential to control
154 developmental patterns at the spatial and temporal level.

155

156 **Acknowledgements**

157 This work was financially supported by the Deutsche Forschungsgemeinschaft (DFG)
158 (Projektnummer 243440351), the Excellence Initiative of the German Federal and State
159 Governments, Scuola Superiore Sant'Anna, the University of Pisa and the Erasmus+
160 programme (Z.N.V.).

161

162 **Author Contributions**

163 D.A.W., J.T.vD. and F.L. designed the experiments. D.A.W., A.B.K., N.C.W.K., K.M.S.P.,
164 N.K.P., Z.N.V., O.P. and F.L. carried out the experiments as described in detail in
165 Supplementary Table 8. D.A.W. and F.L. conducted the statistical analyses. C.G. and J. U. L.
166 provided support for confocal analyses of shoot tissues. D.A.W., J.T.vD. and F.L. wrote the
167 manuscript with inputs by A.B.K, C.G., J. U. L. and O.P. All co-authors read and approved
168 the manuscript.

169 **Author Information**

170 The authors declare no financial or non-financial competing interests. Correspondence and
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271 **Figure legends**

272 **Figure 1. A hypoxic niche is required for proper shoot apical meristem activity.** (**a**)
273 Oxygen concentration through the SAM in the apical basal direction. Colours represent
274 individual measurements. An example of microsensor insertion is shown in the bottom right.
275 (**b**) GFP expression driven by the Hypoxia Responsive Promoter (*pHRPE)* in the
276 Arabidopsis inflorescence meristem. The experiment was repeated three times with similar
277 results. (**c**) Phenotype of plants grown under aerobic and hyperoxic conditions. The
278 experiment was repeated twice with similar results. In total 15 plants for each condition were
279 observed. (**d**) Leaf initiation rate in Arabidopsis plants grown in normoxia and hyperoxia
280 (one-way ANOVA followed by Holm-Sidak post-hoc test, n=15 plants).

281 **Figure 2. ZPR2 is an N-degron pathway substrate.** (**a**) Schematic depiction of the
282 Cys/Arg-branch of the N-degron pathway. (**b**) Amino acid conservation at the N-termini and
283 in the leucine zipper domain of ZPR2-like proteins. (**c**) Nuclear GFP fluorescence in leaf
284 epidermal cells of wild type and *prt6* mutants expressing*MC-ZPR2-GFP* or *MAC-ZPR2-GFP*,
285 in air and after 12 hours of hypoxia. Nuclei were visualised by DAPI staining. The experiment
286 was repeated twice with similar results. (**d**) GFP immunodetection in wild type and *prt6*
287 mutants expressing *MC-ZPR2-GFP* or *MAC-ZPR2-GFP*, in air and after 12 hours of hypoxia.
288 This experiment was repeated three times with similar results.

289 **Figure 3. ZPR2 plays a role in SAM activity.** (**a**) SAM-specific expression of *ZPR2* during
290 vegetative (top) and reproductive (bottom) development using GUS (top left) or GFP
291 reporters. The experiment was repeated twice with similar results. (**b**) Oxygen-dependent
292 accumulation of ZPR2-GUS fusion protein driven by the *ZPR2* promoter. Hyperoxia and
293 hypoxia treatments were performed for 12 hours. The experiment was repeated two times
294 with similar results. **(c)** Shoot phenotype and leaf number of 20-day old wild type, *zpr2-2* and
295 *zpr2-3* plants. The experiment was repeated two times with similar results. (**d**) Leaf initiation
296 rate (leaves d^{-1}) in wild type, *zpr2-2* and *zpr2-3* plants (one-way ANOVA followed by Holm-
297 Sidak post-hoc test n=14 plants, the experiment was repeated twice with similar results).

298 **Figure 4. ZPR2 negatively regulates HD-ZIP III activity.** (**a**) Effect of ZPR2 on the
299 transactivation activity of HD-ZIP III on the *ZPR1* promoter. (one-way ANOVA, Holm-
300 Sidak post-hoc test, n=5 protoplast pools, the experiment was repeated two times with similar
301 results). (**b**) Bimolecular fluorescence complementation of YFP showing interaction of ZPR2
302 with REV, ATHB8 and PHB. The experiment was repeated two times with similar results. (**c**)
303 Differential expression of HD-ZIP III target genes after estradiol (50 μM) mediated *ZPR2*
304 induction in air or hypoxia measured by real-time qRT-PCR. Numerical values and the
305 respective statistical analysis are shown in Supplementary Table S5.

306 **Online methods**

307

308 **Plant materials and growth conditions**

309 *Arabidopsis thaliana* Columbia-0 (Col-0) was used as wild type ecotype. *zpr2-2* (N483079,
310 GK-866D03, previously described by Wenkel et al. 2007)[7] and *zpr2-3* (N835524,
311 SAIL_794_D11.C) and *prt6* [N684039, SALK_051088C defined as *prt6-5* by Riber et al.
312 (2015)][41] T-DNA insertion mutant seeds were obtained from the Nottingham Arabidopsis
313 Stock Centre (NASC). The *pHEC1:GUS* line has been previously described[42]. *Solanum*
314 *lycopersicum* var. Micro-Tom was used for shoot apical oxygen measurement and real-time
315 qRT-PCR, respectively.

316

317 **Growth conditions**

318 Seeds were set in soil (1/3 perlite, 2/3 third peat soil), stratified at 4 °C in the dark for 48 h and
319 subsequently germinated at 16 h photoperiod with 22 °C day/18 °C night. For *in vitro*
320 cultivation, seeds were sowed on agarized half-strength Murashige and Skoog medium
321 supplemented with 1% sucrose and set to germinate at 16 h photoperiod with 22 °C day/18 °C
322 night. Evaluation of the effect of hyperoxia on plant development was performed by growing
323 plants in a continuous flux of high-oxygen [80% (v/v) oxygen in air]. Hypoxic treatments
324 were performed under continuous darkness in Plexiglas boxes continuously flushing an
325 oxygen-modified atmosphere [2% (v/v) O_2/ N_2] for the time indicated in the text or figure
326 legends.

327

328 **Chemicals treatments**

329 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific) and N-(3-
330 Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium
331 Dibromide (FM4-64 dye, Thermo Fisher Scientific) staining was carried out according to the
332 manufactures instructions.

333 Induction of *ZPR2* transgene expression was achieved by 50 µm estradiol, dissolved in
334 ethanol, treatments for 4 hours before the application of hypoxia. An equal amount of ethanol
335 was used as a control.

336

337 **Cloning of the different constructs**

338 Entry clones for *ATHB8* (G24724) and *PHB* (U25105) were obtained from the Arabidopsis
339 Biological Resource Center (ABRC). All other coding sequences were amplified from
340 complementary DNA templates using the Phusion High Fidelity DNA-polymerase (New
341 England Biolabs). Promoter sequences were amplified from wild type (Col-0 ecotype)
342 genomic DNA. All open reading frames were cloned into pENTR/D-TOPO (Thermo-Fisher
343 Scientific). Codon-substitutions were achieved by means of site-directed mutagenesis. The
344 resulting entry vectors were recombined into destination vectors using the LR reaction mix II
345 (Thermo-Fisher Scientific) to obtain the relative expression vectors. A complete list and
346 description of the primers and expression vectors used is provided in Supplementary Tables
347 S6 and S7, respectively.

348 A 31 bp long sequence containing the HRPE element was retrieved from the *LBD41* promoter
349 (-364 to -331 from the initial ATG), repeated five times in tandem and fused to a minimal 35S
350 promoter. This sequence, described in Supplementary Data S1, was synthesized by GeneArt
351 (Thermo Fisher Scientific), inserted into pENTR/D-topo (Thermo Fisher Scientific) and
352 recombined either into the binary vector *pKGWFS7* plasmid[43] or the *p2GWL7*[25] using LR
353 clonase mix II (Thermo Fisher Scientific).

354 **Identification, alignment and phylogenetic analysis of angiosperm ZPRs**

355 Identification of ZPR protein sequences in different sequenced plant species was performed
356 by searching the phytozome database (www.phytozome.net). Protein sequences similar to
357 *Arabidopsis thaliana* ZPR family members were searched using the BLAST algorithm and
358 the ZPR1, ZPR2, ZPR3 and ZPR4 sequences were used as baits. The obtained sequences were
359 multialigned using the MUSCLE[44] algorithm before manual inspection and minor
360 adjustments. Logos of conserved protein motifs were generated by WebLogo[45]. The
361 MEGA7[46] software was used to create a phylogenetic tree. Default settings where used,
362 choosing the WAG model, a Gamma distribution with invariant sites (G+I) for rates among
363 sites and partial deletion to treat missing data and/or gaps. The phylogenetic tree was tested
364 using the bootstrap method with 500 replicates. Conservation of amino acid residues at
365 specific position in the N-terminal sequence of ZPR2-like and ZPR3-like proteins in
366 angiosperms was calculated as percentage of occurrence in the list of sequences considered
367 for the phylogenetic analysis.

368 **Stable plant transformation**

369 Stable transgenic plants were obtained using the floral dip method[47]. T0 seeds were selected
370 for kanamycin, hygromycin and phosphinothricin resistance on agarized MS medium
371 supplemented with the corresponding antibiotic and independent transgenic plants were
372 identified.

373 **Transient protoplast transformation and luciferase assay**

374 A 1.5 kb genomic fragment upstream of the *ZPR1* (*At2g45450*) coding sequence was PCR
375 amplified, cloned into pENTR-D/topo (Thermo-Fisher Scientific) and recombined into
376 *pGreenII-800LUC* to generate a reporter and normalization vector. Non-recombined

377 *p2GW7* vector was used as a negative control. Arabidopsis mesophyll protoplasts were
378 prepared as previously described[48] and transfected using 3 µg plasmid DNA each. Cells were
379 incubated overnight in the dark in a WI solution (0.5 mM mannitol, 20 mM KCl, 4 mM MES
380 pH 5.6 and 10 mM glucose). A dual luciferase reporter assay was performed on protoplasts
381 extract using Dual-Luciferase® Reporter Assay System (Promega) as described previously[23].

382 **Assessment of leaf production rate**

383 The number of visible leaves was determined every two days. The average leaf initiation rate
384 was calculated by dividing the total number of leaves by the days after the first measurement
385 (8 days post germination).

386 **qRT–PCR**

387 Total RNA extraction, DNAse treatment, cDNA synthesis and qRT-PCR analysis were
388 performed as described previously[25].

389 **Confocal imaging**

390 For GFP imaging, leaves from independently transformed plants (two-week old) were
391 analyzed with a Leica DM6000B/SP8 confocal microscope (Leica Microsystems) using 488-
392 nm laser light (20% laser transmissivity), Hybrid detection (HyD), and collected between
393 491- to 551 nm. DAPI was visualized upon laser excitation at 405 nm (2% laser
394 transmissivity, PMT voltage 750 V) and collection at 415-485 nm. FM4-64 staining was
395 detected using a laser excitation at 488 nm (20% laser transmissivity, PMT voltage 500 V)
396 and collection at 650-750 nm. Images were analyzed with the LAS X life science software
397 (www.leica-microsystems.com), with unchanged lookup table (LUT) settings in each channel.
398 For imaging of the vegetative SAM, the meristems of 4-day old seedlings was exposed by
399 peeling off one cotyledon as previously described[49]. Dissection of the inflorescent meristem
400 was performed according to a previously described method[50].

401 **SDS–PAGE and western blotting**

402 Total proteins from leaves excised from three to five individuals belonging to one to three
403 independent insertion lines were extracted as previously described by Huck et al. (2017)[51].
404 Protein samples were separated by SDS–PAGE on 10% acrylamide midigels (Biorad) and

405 then transferred onto a polyvinylidene difluoride membrane (BioRad). The membrane was
406 incubated with a mix of monoclonal anti-GFP antibodies (clones 7.1 and 13.1, Sigma-
407 Aldrich) diluted 1000x fold. Incubations with the antibody and the secondary antibody
408 conjugated to horseradish peroxidase (Agrisera) were performed as described by Beckers et
409 al.[52]. Detection was performed with the Chemidoc MP Imaging System (Biorad) using the
410 Millipore Luminata Forte Western HRP substrate (Merck). Amido black staining was used
411 to confirm equal loading and transfer, according to Goldman et al.[53].

412 **Bimolecular fluorescence complementation assay**

413 Vectors for the expression of *HD-ZIP III* and *ZPR2* fusion to N-terminal or C-terminal *YFP*
414 fragments were generated by recombination of the entry-vectors described above with the
415 destination vectors as reported in Supplementary Table S7 by means of LR recombination
416 using LR clonase II mix (Thermo-Fisher Scientific). Complementation of YFP fluorescence
417 in transiently transformed protoplasts was performed as described previously[54]. Protoplasts
418 were prepared according to a previously described method[25] and transformed using 5 to 10 µg
419 of each BiFC vector.

420 ***In silico* analysis of anaerobic genes in the SAM**

421 RNA-normalized expression levels of anaerobic genes from rosette, roots and SAM were
422 extracted from the eFP browser[55].

423 **Histochemical GUS staining**

424 Histochemical GUS staining was performed according to Jefferson et al.[56]. Imaging of GUS
425 stained plants was performed using a Leica M205 FA stereomicroscope equipped with a Leica
426 DFC450 C camera. Micrographs were analyzed using the ImageJ software[57]. First images
427 were converted into a RGB stack, and then the pixel intensities were inverted. Next, the
428 measurement area, mean grey value and integrated intensity were measured for each
429 individual GUS stained plant. Background values were collected from wild type plants,
430 cleared using 70% (v/v) ethanol. Staining intensity was then calculated according to the
431 follow formula: staining intensity = integrated intensity - (Marked area * mean background
432 grey value).

433 **Quantitative GUS assay**

434 Total proteins were extracted from protoplasts transfected with GUS or firefly luciferase-
435 bearing vectors using Protein Lysis Buffer (Promega). Firefly luciferase activity was
436 measured, as a control for transfection efficiency, using the Luciferase Assay System
437 (Promega) according to the manufacturer's instructions. Protein extracts were incubated in β-
438 glucuronidase activity assay buffer containing 10 mM Tris-HCl pH 8.0, 0.6 mM CaCl, 5 mM
439 4-Nitrophenyl β-D-glucuronide (PNPG, Sigma-Aldrich) for 7 h at 37°C in the dark.
440 Absorbance at 405 nm was measured to record the release of *p*-nitrophenyl. Reactions in the
441 absence of the PNPG substrates were run to correct for background signal.

442 **Microprofiling of O_2 in the shoot apical meristem**

443 Microprofiling of tissue O_2 followed the procedure of Herzog and Pedersen (2014)[58] with few
444 modifications. 4- or 7-day-old seedlings were gently held between two layers of Parafilm with
445 a drop of deionised water to prevent desiccation, fixed in Blue-Tack putty and positioned in a
446 petri dish. Microprofiles of O_2 in the shoot apical meristem were taken using a custom-built
447 Clark-type microsensor with a bevelled tip and a diameter of 3 µm (Unisense A/S, Aarhus,
448 Denmark). The microsensor was connected to a pA meter (Oxymeter, Unisense A/S) and
449 mounted on a motorized micromanipulator (MM33, Unisense A/S). Data acquisition and
450 positioning of the motorized micromanipulator were controlled with Profiling (SensorTrace
451 Suite 2.8). The tip of the microsensor was advanced in steps of 10 µm starting outside the
452 tissue until the target tissue had been completely penetrated. Typically, a complete profile was
453 measured in 10 minutes. Prior to measurements, the sensor was calibrated at air-equilibrium
454 (DI water purged with air, 20.6 kPa pO_2) and at zero O_2 (alkaline DI water with ascorbate).
455 The fine positioning above the target tissue was aided by using a boom-stand dissection
456 microscope. The depth at which the microsensor entered the tissue was followed by
457 microscopic observation. All measurements were carried out at 20 °C in dim light; an initial
458 test showed that light had no significant effect on tissue pO_2 (measured in darkness and at 250
459 µmol photons m^{-2} s^{-1}).

460

461 **Benchmarking of the customized oxygen microsensor against a standard oxygen**
462 **microsensor**

463

464 An oxygen-consuming 2% agar was prepared by adding 2 g of sodium ascorbate to 10 mL of
465 warm liquid agar solution adjusted to pH 11. After cooling to 20 °C, a thin layer of DI water
466 (100-200 µm) was added to the surface of the agar plate. The customized oxygen sensor was
467 compared to a standard microsensor with a tip diameter of 10 µm (OX10, Unisense A/S
468 (Denmark)). Measurements were taken from humid air (20.6 kPa) and into the water-agar
469 interface with steps of 20 µm to a depth of 600 µm. In the graph, 0 on the horizontal axis
470 indicates the transition between air and water-agar. The replicates were taken at different
471 positions on the oxygen-consuming agar plate.

472

473 **Statistics and Reproducibility**

474 The Sigmaplot (Systat) software was used to evaluate significant variations between
475 genotypes or treatments, employing a t-test, one-way or two-way ANOVA where
476 appropriate. Holm-Sidak, Dunn and Tukey post-hoc test were used to assess statistical
477 difference in comparisons after one- or two-way ANOVA. Fisher's Exact tests were carried
478 out using the Graphpad quickcalcs online tool (https://www.graphpad.com/quickcalcs/).
479 The *p* values calculated for each pairwise comparison (Student's t-test, Fisher's exact test
480 and post-hoc tests) are shown in each graph above the line that connects the two sets of
481 data. For all figure legends n represents the number of independent biological replicates.

482 Box plots were generated using BoxPlotR[59]. All boxplot limits represent the 25th and 75th
483 percentiles of each set of data points, the whiskers extend to the lowest and highest data point
484 within 1.5 times the interquartile range of the 25th and 75th quartile. The central line represents
485 the median. All independent data points are displayed in the plots using the R beeswarm
486 package.

487 **Supplementary References for Methods**

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531

532 **Data Availability Statement**

533 Accession numbers of all the Arabidopsis genes analysed in this study are listed in the text or
534 figure legends. All numerical Source Data used to generate the graphs displayed in this report
535 are provided as a supplementary excel file. Seeds of transgenic lines used in this study are
536 available from the corresponding authors upon request. The uncropped version of all
537 gels/blots are provided as SI Figure 1.

538

539 **Extended Data legends**

540

541 **Extended Data Fig. 1: Low-oxygen levels in the Arabidopsis shoot apex overlap with the**
542 **meristematic niche.**

543 (**a**) Comparison of spatial resolution and accuracy of the custom-made Clark-type
544 microsensor used in the present study (red, orange and pink triangles) alongside with the
545 commercially available sensor OX10, Unisense A/S (Denmark) (turquoise, cyan and cobalt
546 blue circles). Oxygen-consuming solid medium was prepared by sodium ascorbate oxidation.
547 The data set shows that in 2% agar with a steep gradient in dissolved oxygen from
548 atmospheric equilibrium to anoxia, the two sensors show identical responses. Five
549 independent profiles were recorded for each microsensor type. (**b**) Depiction of the

550 experimental setup adopted to measure the oxygen concentration profiles in the apical-basal
551 direction (left) and radial direction (right). (**c**) Oxygen concentration profile in the apex of 4-
552 day old Arabidopsis seedling. This experiment was repeated two times with similar results.
553 (**d**) Tracking of the sensor insertion pattern reconstructed after the oxygen profile (e) was
554 taken. The shoot apical meristem was visualized by confocal microscopy following FM4-64
555 staining of plasma membranes. (**e**) Oxygen concentration profile obtained by inserting the
556 Clark-type microsensor laterally through the shoot apical meristem of 7-day old Arabidopsis
557 plants in the shoot apical region (1), at the junction of cotyledon vasculature (2) and below the
558 junction (3). The experiment was repeated three times with similar results. A photo of the
559 experimental setup is shown within the plot frame (bottom right). (**f**) Position of the sensor
560 insertion points as identified by optical microscopy (top) and localization of the *CLAVATA3*
561 expression domain, reported by GUS staining of plants expressing *pCLV3:GUS* in 4-day old
562 plants (bottom).

563

564 **Extended Data Fig. 2: Transcripts belonging to the core hypoxia-inducible genes are**
565 **specifically enriched in shoot but not in root meristem tissues.** (**a**) Pie-charts representing
566 the relative abundance of differentially expressed genes within the core hypoxia-inducible set[1]
567 in comparison between shoot apical meristem (SAM) and juvenile leaf tissues, and root
568 meristem (RM) and elongation zone/maturation zone of the root. Absolute expression levels,
569 standard error, ratios and statistics are shown in Supplementary Table S1 and S2 for shoots
570 and roots respectively. (**b**) Schematic representation of SAM and RM cell types. (**c**) Heatmap
571 showing the expression levels of core-hypoxia inducible genes in the SAM and RM cell types
572 depicted in (b). Absolute expression levels were retrieved from the Arabidopsis EFP browser
573 and are provided in Supplementary Table S3.

574

575 **Extended Data Fig. 3: Low oxygen conditions in the shoot apex induce molecular**
576 **hypoxic responses.**

577 (**a**) Oxygen responsiveness of a synthetic construct consisting of five repeats of the
578 Hypoxia Responsive Promoter Element (HRPE), named there *pHRPEx5:GFP-GUS*
579 reporter. The experiment was repeated twice with similar results. (**b**) and (**c**) Quantification
580 of the relative staining intensity of the *pHRPEx5:GFP-GUS* reporter at 21, 10, 5 (b) and

581 80% O_2 (c) shows that this construct effectively reports tissue oxygen status. Statistical
582 analysis was applied as follows: (b), one-way ANOVA followed by Tukey post-hoc test,
583 (c) two-sided t-test. (**d**) Real time qRT-PCR was used to measure the expression of hypoxia-
584 inducible genes *Plant Cysteine Oxidase 1* (PCO1), *Wound Induced Protein 4 (WIP4),*
585 *Hypoxia Responsive Attenuator 1 (HRA1), Plant Cysteine Oxidase 2 (PCO2)*, *Hemoglobin*
586 *1(HB1)*, *Pyruvate Decarboxylase 1* (*PDC1)*, *Alcohol Dehydrogenase* (*ADH)* in leaves and
587 shoot apices, after normoxic and hyperoxic treatments (80% O_2, 6 h). The higher expression
588 of these mRNAs in the SAM, as compared to the leaf samples, was repressed by hyperoxia. A
589 two-way ANOVA followed by Tukey post-hoc test was applied to assess statistical
590 significance of the observed differences.

591

592 **Extended Data Fig. 4: Hypoxic conditions are maintained in reproductive meristems.**

593 (**a**) Oxygen concentration profiles obtained inserting the Clark-type microsensor in the apical-
594 basal direction through the inflorescence meristem of 5-week old Arabidopsis plants. The
595 inlet shows a photo of the actual experimental setup with the insertion of the microsensor
596 inside the inflorescence meristem. This experiment was repeated independently three times
597 with similar results. (**b**) Green fluorescent signal in inflorescence and floral meristems of
598 plants transformed with the hypoxia reporters *pPCO1:GFP-GUS* and the *pPCO2:GFP-GUS*
599 characterized as specifically hypoxia responsive in Weits et al.[25]. The experiment was
600 repeated two times with similar results.

601

602 **Extended Data Fig. 5: A hypoxic niche at the shoot apex is a common feature of plants.**

603 (**a**) The customized Clark-type microsensor was used to measure oxygen profiles through the
604 shoot apex of 1-week old *Solanum lycopersicum* var. Micro-Tom plants, in the apical basal
605 direction. These measurements show the presence of an oxygen gradient in the shoot apex of
606 this plant species. The experiment was repeated two times with similar results. (**b**) Photo
607 showing the insertion of the micro-electrode inside the tomato SAM. (**c-d**) Overlay of oxygen
608 profiles [shown as cyan and red in (a), respectively] and micrographs of the SAM tissues after
609 FM4-64 membrane staining that show the actual penetration of the sensor. The puncture in the
610 centre of the meristem and the concomitant accumulation of FM4-64 shows the penetration of
611 the sensor into the tissue. The experiment was repeated four times with similar results, two

612 examples are shown in c and d. (**e**) Similarly to Arabidopsis, typical hypoxia marker genes
613 *Alcohol Dehydrogenase 2* (*ADH2*), *Pyruvate Decarboxylases* (*PDC1* and *3*), *Plant Cysteine*
614 *Oxidase 2* (*PCO2*) and *Phytoglobin 1* (*HB1a*) are higher expressed in SAM-enriched tissues
615 than in juvenile leaves of 2-week old plants of *Solanum lycopersicum* var. Micro-Tom.
616 *Pyruvate Decarboxylase 4 (PDC4)* does not exhibit the same pattern. These results indicate
617 that SAM cells experience hypoxic conditions. Real time qRT-PCR was used to measure the
618 expression of hypoxia-inducible genes (two-sided t-test, n=4 pools of 5 plants).

619

620 **Extended Data Fig. 6: ZPR2-type proteins are distinguished by a variable N-terminal**
621 **domain with a conserved Cys residue in the penultimate position.**

622 (**a**) Multi-alignment of ZPR-type sequences from eight different angiosperm species
623 (*Aquilegia coerulea, Amborella trichopoda, Arabidopsis thaliana, Daucus carota, Medicago*
624 *truncatula, Oryza sativa, Populus trichocarpa, Solanum lycopersicum,* and *Sorghum bicolor*).
625 At least one ZPR2-type and one ZPR3-type sequence was identified in all species considered.
626 All ZPR2-type proteins have a Cys residue at the second position of the N-terminal domain.
627 Amino acid position is displayed on top of each alignment, using AtZPR2 as a reference. (**b**)
628 Molecular Phylogenetic analysis by Maximum Likelihood method. The tree with the highest
629 log likelihood (-1500.12) is shown. The percentage of trees in which the associated taxa
630 clustered together is shown next to the branches. The tree is drawn to scale, with branch
631 lengths measured in the number of substitutions per site. The analysis involved 28 protein
632 sequences (listed in Supplementary Table S4).

633

634 **Extended Data Fig. 7: N-terminal tagging of proteins with a ZPR2 sequence confers**
635 **degradability by the N-degron pathway.**

636 (**a**) Relative luciferase activity of a chimeric protein consisting of the whole *ZPR2* coding
637 sequence fused to the N-terminus of a firefly luciferase (*ZPR2-PpLUC*). This construct was
638 transfected together with a second one, bearing a renilla luciferase gene driven by the same
639 35S CaMV promoter, into Arabidopsis mesophyll protoplasts. Renilla luciferase activity was
640 used as a normalization control (one-way ANOVA followed by a Tukey post-hoc test, n=6, 4,
641 5 protoplast pools for MC-ZPR2, MAC-ZPR2 and MCZPR2 in *prt6* respectively). (**b**)
642 Relative GUS activity of a *gZPR2-GUS* construct expressed in Arabidopsis protoplasts. A

643 *35S:PpLuc* reporter was co-transformed to equalize for transfection efficiency. The addition
644 of an Ala residue before the Cys2 led to enhanced stability of both reporter constructs, while
645 the expression of the wild type ZPR2 protein fusions in the *prt6* mutant background also
646 showed enhanced protein abundance. One-way ANOVA followed by a Tukey post-hoc test,
647 n=8, 5, 6 protoplast pools for MC-ZPR2, MAC-ZPR2 and MCZPR2 in *prt6* respectively. (**c**)
648 Quantification of the relative staining intensity of GUS stained plants expressing a
649 *pZPR2:ZPR2-GUS* construct at 21, 2, and 80% O_2. An example of GUS staining at each
650 oxygen concentration is shown in Fig. 3b. Images of GUS stained plants were converted to
651 inverted grey scale images and the staining intensity was measured using ImageJ. Wild
652 type plants destained in ethanol were used to correct for the background signal. Average
653 relative staining intensity was calculated by comparing the corrected staining intensity at
654 each O_2 concentration by the corrected staining intensity at 21% O_2. These results show
655 that ZPR2 stability in the SAM depends on the oxygen availability (one-way ANOVA
656 followed by a Holm-Sidak post-hoc test, n=10, 7, 8 plants for 2, 21, and 80% O_2
657 respectively). (**d**) Relative mRNA level of the genotypes used for immune-detection of GFP
658 in Fig. 2d under aerobic and hypoxic conditions (2% O_2) measured by qRT-PCR. Two
659 biological replicates from two independent lines were used in the case of *35S:MC-ZPR2-GFP*
660 and *MAC-ZPR2-GFP* lines. Four biological replicates were instead used in the case of *MC-*
661 *ZPR2-GFP* in the *prt6* background. The effect of hypoxia treatment versus aerobic conditions
662 was evaluated by two-way ANOVA (p value=0.342, n=4 pools of three plants). The results of
663 the qRT-PCR analysis exclude regulation by hypoxia at the transcriptional level and,
664 combined with the immunoblot analysis, support the existence of a control checkpoint at the
665 post-transcriptional level. (**e**) Effect of ZPR2 and ZPR2-GUS on the transactivation activity of
666 REVOLUTA on the *ZPR1* promoter. These data indicate that ZPR2 is not able to repress
667 REVOLUTA activity when fused with a GUS reporter protein at its C-terminus. Asterisks
668 indicate a statistically significant difference (one-way ANOVA, followed by Holm-Sidak
669 post-hoc test, n=5 protoplast pools).

670

671 **Extended Data Fig. 8: Loss of *ZPR2* expression reduces the leaf initiation rate, while**
672 **ectopic overexpression of *ZPR2* abolishes SAM activity in an oxygen-dependent manner.**
673 (**a**) Schematic depiction of the *ZPR2 (AT3G60890)* gene showing the position of two T-DNA
674 insertions (Sail and GABI-kat collections). The relative annealing site of the primers used in
675 (b) is shown using arrows in the schematic view in the top panel. (**b**) The homozygous status

676 of T-DNA insertions within the intron of *AT3G60890* in the NASC accessions N483079
677 (*zpr2-2*, GK-866D03) and N835524 (*zpr2-3*, Sail_794_D11) was tested by PCRs using the
678 combinations of primers indicated in (a). Genomic wild type (Col-0 ecotype) DNA and
679 double distilled water were used as controls. The experiment was repeated two times with
680 similar results. (**c**) Amplification of the entire *ZPR2* coding sequence in the wild type, *zpr2-2*
681 and *zpr2-3* genotypes. The experiment was repeated two times with similar results. (**d**)
682 Relative expression of *ZPR2* in wild type, *zpr2*-2 and *zpr2-3* measured by qRT-
683 PCR.Statistical significance of differences was tested by one-way ANOVA followed by a
684 Holm-Sidak post-hoc test, n=5 pools of three plants. (**e**) Progression in leaf number in wild
685 type and *zpr2* T-DNA insertion mutants. The number of leaves, including cotyledons, was
686 counted every second day since the emergence of the first pair of true leaves. Data are
687 presented as means, error bars represent standard deviation, n=15 plants. (**f**) Shoot phenotype
688 of wild type, *zpr2-3* and *pZPR2:ZPR2-FLAG* in *zpr2-3* plants. At the growth condition used,
689 the rosette of 4-week old *zpr2-3* plants was smaller than the wild type. (**g**) Leaf initiation rate
690 in wild type, *zpr2* and *pZPR2:ZPR2-FLAG* in *zpr2-3* plants. (one-way ANOVA followed by
691 Dunn's post-hoc test, n=41, 27, 20, 19 plants for wild type, *zpr2* and *zpr2-3 pZPR2:ZPR2-*
692 *FLAG-1* and line 2 respectively). (**h**) Phenotype of wild type Arabidopsis and *35S:ZPR2-GFP*
693 plants grown in plates containing agarized MS (half-strength) medium supplemented with 10
694 g L^{-1} sucrose under aerobic or hypoxic (2% O_2) conditions for 20 days, followed by 5 days of
695 recovery in normoxia. *35S:ZPR2-GFP* often showed termination of meristem activity and the
696 formation of a pin-like structure. The experiment was repeated two times with similar result.
697 (**i**) Percentage of shoot meristem termination (blue) or meristem progression (green) events in
698 wild type and *35S:ZPR2-GFP* plants after 5 days of recovery from hypoxic growth. The
699 number of plants displaying either phenotype is reported, in white, inside the bar. The
700 frequency of SAM termination in *35S:ZPR2-GFP* plants increased following hypoxic
701 treatments. A two-tailed Fisher Exact test was used to compare wild type and *35S:ZPR2-GFP*
702 grown and aerobic and hypoxic conditions.

703

704 **Extended Data Fig. 9: Repression of HD-ZIP III target genes by *ZPR2* induction and**
705 **stabilization occurs in the shoot apex.**

706 (**a**) Schematic representation of the construct providing estradiol-inducible expression of
707 *ZPR2* and following protein stabilization under hypoxic conditions. (**b**) Separate and

708 combined effect of estradiol (50 µM) application for 4 h before exposure to 2% O_2 for 24 h on
709 the expression of a *GUS* reporter under the control of *pHEC1* (*HECATE 1*) and *pPSK5*
710 (*PHYTOSULFOKINE 5 PRECURSOR*) promoters in 6-day old Arabidopsis seedlings also
711 expressing an estradiol-inducible *ZPR2* construct. Seeds of these genotypes were obtained as
712 F1 offspring generated by crossing homozygous *promoter:GUS* lines with homozygous
713 estradiol-inducible *ZPR2* (*pMDC7:ZPR2*) plants. The observation was repeated twice, a
714 reduction in *pPSK5* or *pHEC1* activity by combined *ZPR2* induction and hypoxia was
715 observed in a total of 8/12 and 15/20 plants, respectively. (**c**) Effect of estradiol-mediated
716 induction of *ZPR2* and its stabilization by hypoxia on *pTAA1:GUS* staining in 5-day old wild
717 type and transgenic *pMDC7:ZPR2* plants. Twenty-four hours of hypoxia, but not estradiol (50
718 µM), was sufficient to repress *pTAA1*-driven *GUS* expression in the wild type background,
719 likely via stabilization of the endogenous ZPR2 protein (2/3 plants). The hypoxia treatment
720 also inhibited expansion of the first pair of true leaves. Stimulated *ZPR2* expression in the
721 *pMDC7:ZPR2* background further decreased *pTAA1:GUS* staining (3/3 plants). This
722 experiment was performed once.

723

724 **Extended data Fig. 10. ZPR2 is required to repress HD-ZIP III target genes. (a)** The
725 expression of HD-ZIP III target genes was measured using real time qRT-PCR in apices of
726 one-week old *zpr2-3* mutants and wild type plants. These results show that the expression of
727 five HD-ZIP III target genes (*PSK5, ATH1, ZPR1, AMP1* and *POL*) is significantly increased
728 in *zpr2-3* insertion mutants (two-sided t-test, n=10 plants). **(b)** GUS expression under control
729 of the *MAX2* promoter in wild type and *zpr2-3* background genotypes. Restoration of wild
730 type background was obtained by PCR screening (for *zpr2-3* insertion) and antibiotic
731 resistance (for the *pMAX2:GUS* construct) in the F2 offspring of a cross between a
732 *pMAX2:GUS zpr2-3* plant and a wild type (Col-0) plant. The observation was repeated two
733 times. In total, induction of *pMAX2:GUS-GFP* in *zpr2-3* mutants was observed in 16/20
734 plants. (**c**) Hypothetical model describing how local hypoxia drives SAM activity by
735 regulating HD-ZIP III transcription factors via ZPR2.

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