Review

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Molecular oxygen as a signaling component in plant development

Author for correspondence:
Daan A. Weits
Tel: +39 050 881914
Email: d.weits@santannapisa.it

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Daan A. Weits[1], Joost T. van Dongen[2] and Francesco Licausi[1,3]

[1]Plantlab, Institute of Life Sciences, Scuola Superiore Sant'Anna, Pisa 56010, Italy; [2]Institute of Biology I, RWTH Aachen University, Aachen 52074, Germany; [3]Biology Department, University of Pisa, Pisa 56126, Italy

Contents

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Summary

While traditionally hypoxia has been studied as a detrimental component of flooding stress, the last decade has flourished with studies reporting the involvement of molecular oxygen availability in plant developmental processes. Moreover, proliferating and undifferentiated cells from different plant tissues were found to reside in endogenously generated hypoxic niches. Thus, stress-associated acute hypoxia may be distinguished from constitutively generated chronic hypoxia. The Cys/Arg branch of the N-degron pathway assumes a central role in integrating oxygen levels resulting in proteolysis of transcriptional regulators that control different aspects of plant growth and development. As a target of this pathway, group VII of the Ethylene Response Factor (ERF-VII) family has emerged as a hub for the integration of oxygen dynamics in root development and during seedling establishment. Additionally, vegetative shoot meristem activity and reproductive transition were recently associated with oxygen availability via two novel substrates of the N-degron pathways: VERNALISATION 2 (VRN2) and LITTLE ZIPPER 2 (ZPR2). Together, these observations support roles for molecular oxygen as a signalling molecule in plant development, as well as in essential metabolic reactions. Here, we review recent findings regarding oxygen-regulated development, and discuss outstanding questions that spring from these discoveries.

I. Introduction

The oxygen-rich atmosphere of our planet is a consequence of photosynthesis carried out by phytoplankton in the oceans and land plants on Earth's surface. However, molecular oxygen represents a double-edged sword for plants: it is essential for ATP production via mitochondrial respiration, but it also participates in the production of highly reactive molecules and competes with carbon dioxide as a substrate for the enzyme Rubisco. Oxygen concentrations therefore require careful homeostasis, and it is not surprising

that plant cells mount up adaptive responses when they face variable oxygen provision. A common response to hypoxia has been identified in higher plants, including the induction of fermentation, NO metabolism and protective proteins (Mustroph *et al.* 2009). Nonetheless, it is the adoption of tissue and species-specific mechanisms that ensures tolerance in individuals that live in habitats more likely to experience oxygen shortage (Bailey-Serres & Voesenek, 2008). Typically, oxygen concentrations in plant tissues drop when its consumption by cellular respiration is greater than its rate of diffusion from the atmosphere. Specific environmental conditions, such as flooding, ice encasement or artificially manipulated atmospheres, especially affect oxygen availability to plant cells. However, endogenously generated oxygen gradients also commonly occur in plants, where the anatomy and physiology of the specific tissue determines oxygen release, retention or diffusion (van Dongen & Licausi, 2015). For instance, in ripe fruits, seeds, and roots, where photosynthesis is limited or non-existent, oxygen concentrations have been documented to fall below 5% (van Dongen *et al.*, 2003; Borisjuk & Rolletschek, 2009; Ho *et al.*, 2010). Moreover, despite the apparent limitation of oxygen for aerobic metabolism, these conditions actually represent a physiologically normoxic level for the tissues mentioned here. Borrowing terms from the nomenclature currently in use in animal biology, continuous and homeostatic hypoxia in these tissues can be described as 'chronic' hypoxia, while a drop in the oxygen concentrations upon a change in the environment or a rapid induction of oxygen consumption can be termed 'acute' hypoxia (see Box 1).

II. Meristems enclosed in chronic hypoxic niches

The existence of oxygen gradients in plant tissues and the identification of regions characterized by low oxygen concentrations have been reported since the early twentieth century (J. R. Magness, 1920). These initial observations were later refined by the development of Clark electrodes and oxygen optodes (Clark *et al.*, 1953; Severinghaus & Astrup, 1986; Ast *et al.*, 2012). Only recently, however, with the availability of molecular reporters and micrometric sensors, have researchers started to explore cell niches of a smaller size. Indeed, two reports have now identified chronic low oxygen conditions in zones that harbour the stem cells that sustain growth and development: the meristems (Fig. 1) (Shukla *et al.*, 2019; Weits *et al.*, 2019). Endogenous and synthetic promoters activated by hypoxia were found to be active in both shoot apices and lateral root primordia (LRP). Moreover, oxygen concentrations below 5% (v/v) were directly measured in Arabidopsis and *Solanum lycopersicum* shoot apical meristems by means of an oxygen microsensor with a diameter of 3–5 μm.

Notably, oxygen microprofiling of shoot apices showed a steep drop below 5% O_2 in the meristem, over a remarkably short distance. Such a steep oxygen gradient could arise from high respiration rates of the meristematic cells (Fig. 2a), or could hint at a yet to be identified oxygen barrier covering the meristematic niche (Fig. 2c). Alternatively, high respiration rates in the outer cells of a niche could lead to internal low oxygen conditions (Fig. 2b). The lack of air spaces for gas exchange between the densely packed small cells of the meristems likely also imposes a limit to oxygen diffusion within this tissue. Moreover, several days after germination, shoot apical meristems are covered by several growing leaves, which could further limit the diffusion of atmospheric oxygen to the shoot apical meristem (SAM). For instance, Meitha *et al.* (2015) observed that dormant grapevine bud meristems are covered by several lignified bracts that protect the bud during winter, but also establish a barrier to oxygen diffusion. Removal of the outer bracts led to an increase in the oxygen concentration of the bud, although the core of the bud remained hypoxic, suggesting that the bracts impose a diffusion barrier but are not solely responsible for maintaining a low oxygen environment in the meristematic region. Additionally, in Arabidopsis, low oxygen concentrations were already observed in apices of young seedlings that have only formed two leaf primordia that do not cover the meristem (Weits *et al.*, 2019). Therefore, it appears more likely that low oxygen conditions are due to metabolic or morphological features of the meristem itself, rather than the organs that cover it.

In roots, properties inherent to the LRP seem also sufficient to cause a drop in the oxygen concentration as described for the SAM, since induction of core hypoxia responsive genes was found in Arabidopsis LRPs cultured on solid agar plates where roots are in direct contact with the air in the Petri dish (Shukla *et al.*, 2019). Additionally, one should consider that, when plants grow in soil, LRP emerge in a substrate whose oxygen diffusion rates are strongly affected by the porosity and the water content of the soil (Lemon & Erickson, 1952). This may impose a steeper oxygen gradient upon LRP than observed under sterile culture conditions in the lab. Surprisingly, hypoxia-responsive genes were not found to be induced in the root apical meristem (RAM) when plants were grown under aerobic conditions (Eysholdt-Derzsó & Sauter, 2017; Shukla *et al.*, 2019; Weits *et al.*, 2019). This hints at a divergence in oxygen-dependent regulation between LRP and RAM. It is possible that low oxygen conditions are only required during the establishment of a root meristem, but not to maintain it. Alternatively, oxygen gradients might be maintained in the RAM, although they either do not reach the critically low conditions that induce hypoxia-responsive genes in Arabidopsis, or RAM-specific repressors locally prevent the expression of anaerobic genes in the root tip.

Box 1 Distinction between acute and chronic hypoxia

Acute hypoxia: drop in oxygen availability as a result of limited environmental provision, such as during flooding, or greatly enhanced oxygen consumption. Perceived by the plant as stress, hypoxia may be detrimental for survival if it persists. Adaptive responses include escape or quiescence growth strategies, ROS scavenging and a shift towards fermentative metabolism.
Chronic hypoxia: constitutive and non-stressful oxygen concentrations locally maintained at lower levels than the rest of the plant body. Generated by specific features of the plant tissue, such as high respiration rates or barriers to oxygen diffusion. Chronic hypoxic niches host the SAM and LRP, where oxygen levels act as a developmental signalling cue.

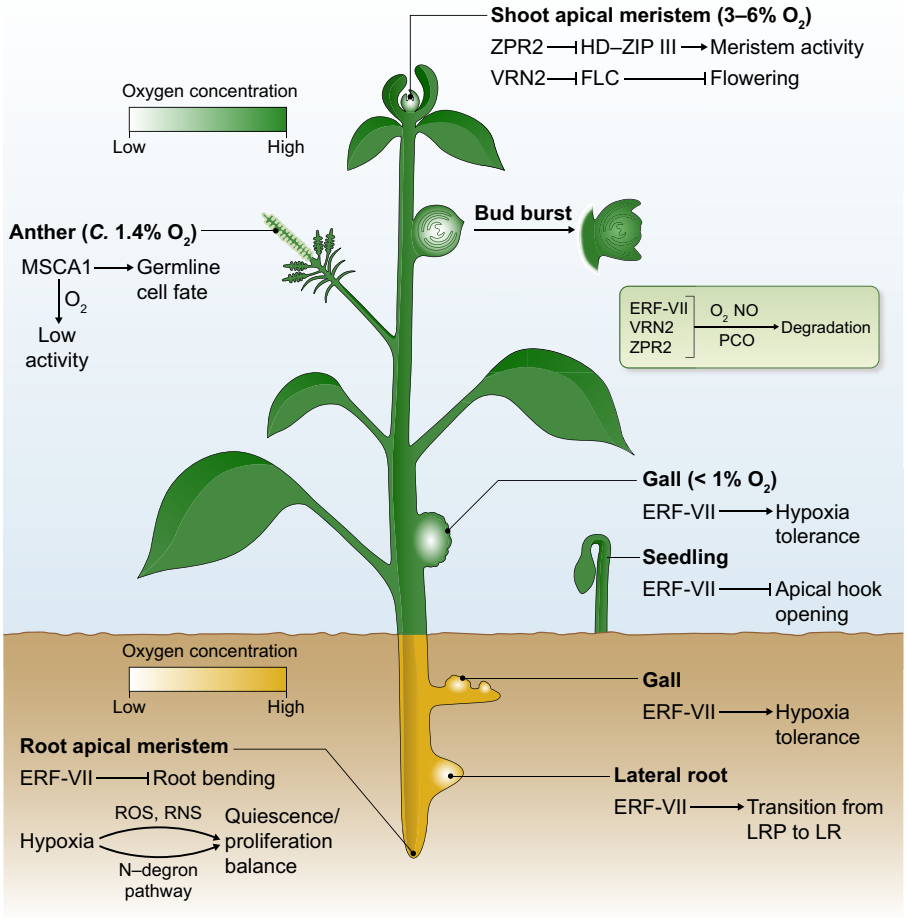

Fig. 1 Overview of developmental processes affected by oxygen availability in plants. Oxygen gradients are schematically indicated in the figure by green (shoot) or yellow (root) colour shades. For each tissue, the molecular mechanism that governs oxygen-dependent development is described with a simplified scheme. Chronic hypoxia has been observed in the shoot apical meristem (SAM), in Arabidopsis lateral root primordias (LRPs), in dormant grape buds, in pathogen-induced galls and during germ cell formation in maize anthers. In the latter, hypoxia regulates germline cell fate through MALE STERILE CONVERTED ANTHER 1 (MSCA1). Although oxygen concentrations during seedling establishment have not yet been assessed, it has been shown that hypoxia regulates apical hook opening through proteolysis of group VII of the Ethylene Response Factor (ERF-VII) family. Recent evidence indicates that the apical root meristem is not hypoxic, although a role for ERF-VII has been found to direct root growth upon variable soil oxygen availability. In the quiescent centre (QC), it has been proposed that oxygen plays a role to regulate the balance between quiescence and cell proliferation, either by affecting reactive oxygen/nitrogen species (ROS/RNS), or via proteolytic activity of the N-degron pathway. The presence of chronic hypoxic conditions during lateral root (LR) formation promotes ERF-VII stability and thereby regulates the transition from LRP to LR. A hypoxic niche at the shoot meristem promotes stability of VERNALIZATION 2 (VRN2) and LITTLE ZIPPER 2 (ZPR2), regulating reproductive and vegetative development through their respective targets FLOWERING LOCUS C (FLC) and class III homeodomain leucine zipper (HD-ZIP III) proteins. ERF-VII also promotes adaption to hypoxia in pathogen-induced galls, thus contributing to tumour growth. The transition from dormant to bud burst has been shown to concur with oxygenation of the tissue, although the mechanism underlying this phenomenon remains to be elucidated.

Direct oxygen measurements or the development of novel oxygen biosensors are required to assess this.

Previous reports also provide a potential link between the vasculature and hypoxia. Indeed, in *Ricinus communis*, oxygen concentrations were shown to drop to 7% O_2 in the vasculature of the stem, and low oxygen (*c.* 5% O_2) pressure has also been measured in the steel of *Zea mays* roots (Armstrong *et al.*, 1994; van Dongen *et al.*, 2003). Primary vasculature originates from both root and shoot apices, whereas secondary phloem and xylem arise from lateral meristems in the vascular cambium of roots and stems. Here, local auxin and cytokinin gradients specify the stem cell organizers, showing a surprising similarity to apical meristems (Miyashima *et al.*, 2019; Smetana *et al.*, 2019). The observation that hypoxic conditions are associated with vascular tissues now paves the way to investigate oxygen tensions at the cellular scale of the vascular cambium.

III. Energy metabolism in low oxygen niches

The measurement of low oxygen concentrations in specific plant tissues, including meristems, stimulates two questions: first, are hypoxic niches within the plant actively maintained by developmental programmes? And, consequently, how do cells cope with chronically low oxygen concentrations in these hypoxic niches?

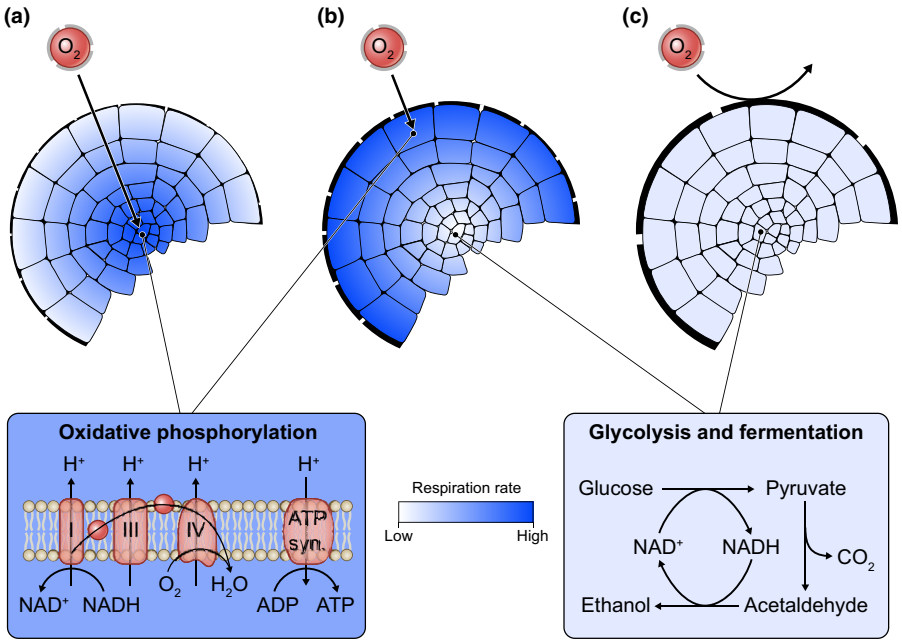

Fig. 2 Hypothetical models for the origin of chronic hypoxia in plant cell niches. A generic structured cell mass is depicted and coloured in shades of blue to indicate relative respiratory activity. (a) High respiratory activity of the central-localised cells could drive hypoxia. (b) Oxygen consumption by the outer niche cells limits oxygen diffusion to the niche core. (c) A physical barrier to oxygen diffusion promotes the establishment of hypoxia in the entire niche. Proposed metabolic pathways in the different regions of the niche are indicated at the bottom of the figure: oxidative phosphorylation via the mitochondrial electron transport chain that uses oxygen as the terminal electron acceptor (left) or glycolytic production of ATP sustained by NAD + regeneration via fermentation (right).

Plant meristems are typical examples of heterotrophic shoot tissues: their plastids are mostly undifferentiated, and expression of genes related to photosynthesis (e.g. genes encoding for Rubisco subunits) are maintained in an off-state (Mandel *et al.*, 1995; Fleming, 2006). Furthermore, exposure of the SAM to light through the removal of leaf primordia, did not lead to Chl formation or the expression of Rubisco subunit transcripts, indicating that meristems are genetically programmed to avoid photosynthesis (Fleming *et al.*, 1996). Thus, meristems have no means of releasing oxygen, and a high rate of oxygen consumption via mitochondrial respiration likely concurs to establish local hypoxia. As discussed in the previous paragraph, oxygen diffusion barriers, whose nature remains enigmatic, may also contribute to maintain persistently low oxygen concentrations in meristems.

To the best of our knowledge, no extensive metabolomic surveys have been carried out on plant meristems. However, based on their hypoxic state and lack of photosynthetic activity, we speculate that energy demand in chronic hypoxic niches is met via two, non-mutually exclusive, metabolic strategies (Fig. 2). First, even low oxygen concentrations in hypoxic niches could maintain ATP production via oxidative phosphorylation if there is a continuous flux of oxygen to the niche (Fig. 2a). Indeed, oxygen consumption via respiration can drive oxygen diffusion along a steep gradient from the atmosphere to the hypoxic meristem. However, local oxygen availability in meristems might be insufficient to support respiration and in this scenario a significant amount of ATP would be produced by glycolysis coupled to fermentation (Fig. 2b,c). In support of this latter hypothesis, core hypoxic genes, including those coding for fermentative enzymes alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) are expressed in the SAM and LRP (Shukla *et al.*, 2019; Weits *et al.*, 2019). Compared to oxidative phosphorylation, substrate-level phosphorylation produces few ATP molecules per unit of glucose. However, glycolysis does have the potential to produce ATP at a faster rate when reserves are adequate, and it does not lead to the potential production of highly reactive species, which may be favourable for cell proliferation (Brand & Hermfisse, 1997; Pfeiffer *et al.*, 2001; Bui & Thompson, 2006; Ito & Suda, 2014; Kerpen *et al.*, 2019). If this hypothesis holds true, anaerobic metabolism in the SAM and LRP would require a rapid delivery of carbon reserves to these tissues.

Assessment of steady state concentrations or fluxes of metabolites in meristems was traditionally limited by the need for dissection of the tissue before mass spectrometry, restricting the spatial resolution of this method. In addition, isolation of specific meristem cell types by protoplasting and fluorescent cell sorting would also unavoidably affect the concentration of the metabolites in such analysis. However, new advances in single cell metabolomics using matrix-assisted laser desorption ionization (MALDI), secondary ion mass spectrometry (SIMS) or even techniques for live analysis, such as Probe ESI-Mass Spectrometry open a new avenue for the study of meristem metabolism (Boggio *et al.*, 2011; Lanni *et al.*, 2012; Gong *et al.*, 2014). State of the art specific biosensors, which detect analytes in live organisms, represent a valid alternative for the imaging of metabolites (Okumoto *et al.*, 2012; Liu *et al.*, 2015). Biosensors present several major advantages: first, their use circumvents the need to isolate the tissue for analyses; secondly, they can be genetically encoded, which precludes the need for

invasive delivery, and the use of fluorescent reporters allows live imaging in the tissue with high spatial and temporal resolution. Therefore, a combination of metabolic fluorescent biosensors would be suitable to study the metabolic activity of hypoxic niches, although these can only provide information for a very limited number of molecules simultaneously.

IV. Association between cellular proliferation and chronic hypoxia

Besides meristems, other actively proliferating plant tissues, such as pathogen-induced galls and *in vitro*-induced calli, were also shown to manifest chronic hypoxia. Crown galls are genetically reprogrammed by *Agrobacterium tumefaciens* to produce opine metabolites, and these tumours turn hypoxic (< 1% O_2) and induce low oxygen responsive genes within 7 d (Deeken *et al.*, 2006; Kerpen *et al.*, 2019). This is consistent with reports by Gravot *et al.* (2016), who also observed the induction of hypoxia-responsive genes, such as *ADH1*, *PDC1* and *PDC2* after infection with *Plasmodiophora brassicae*, another gall producing pathogen. In crown galls and clubroots, ERF-VII TFs are required to sustain cell proliferation, likely by promoting metabolic adaptation to chronic hypoxia. One could speculate that opine anabolism, enforced by the parasite, stimulates respiratory oxygen consumption to an extent that causes hypoxia in the infected tissue. However, the observation of similarly low oxygen concentrations in undifferentiated calli, induced by cytokinin and auxin supplementation, rather suggests that the energy production required to sustain cell proliferation drives rapid oxygen consumption (Kerpen *et al.*, 2019). Also, in animals rapid cell proliferation in solid tumours is associated with the generation of hypoxic conditions. Moreover, several studies pointed at a correlation between metastatic progression and hypoxia (Rankin & Giaccia, 2016). Analogous to the function of ERF-VII in galls, hypoxia-dependent stabilization of the HYPOXIA-INDUCIBLE FACTOR 1α (HIF-1α), promotes metabolic adaptation to hypoxia in tumour cells through strong activation of the glycolytic pathway (Masoud & Li, 2015). HIF-1α also fulfils specific developmental roles by promoting the formation of new blood vessels and by inducing the expression of pro-oncogenes such as epidermal growth factor. Thus, both animal and plant tumours exploit the respective oxygen sensing pathways for adaptation to hypoxia caused by their own metabolism.

V. Molecular mechanisms involved in oxygen-dependent development

At first sight, chronic hypoxic conditions in meristems may appear to set a counterintuitive limit on growth, because reduced oxygen availability would affect energy production via oxidative phosphorylation. However, when hypoxia in the SAM or LRP was relieved through hyperoxia treatments, meristem activity actually slowed down (Shukla *et al.*, 2019 and Weits *et al.*, 2019). This suggests that low oxygen conditions in these meristems acts as a favourable condition for plant development. To search for integrators of variable oxygen concentrations into developmental programmes, recent studies have been directed to oxygen-dependent substrates of the Arg/N-degron pathway (Varshavsky, 2019). Since several reviews have been written to discuss the N-degron and C-degron pathways for protein degradation, our discussion will be brief (Dissmeyer, 2019; Holdsworth *et al.*, 2019; Varshavsky, 2019). These biochemical pathways determine protein half-life on the basis of the amino acid residues exposed at the N- and C-termini (Bachmair *et al.*, 1986; Koren *et al.*, 2018; Lin *et al.*, 2018). Primary destabilizing amino acids, such as Arg, provide direct substrate specificity for RING/U-box E3 ligases, which stimulate polyubiquitination and subsequent degradation of the targets by the proteasome complex (Varshavsky, 2011). Secondary and tertiary residues, instead, require post-translational modification before they can promote proteolysis. In this frame, when methionine peptidases excide N-terminal methionine and expose a cysteine, this residue can be oxidized to cysteine-sulfinic acid in the presence of oxygen by plant cysteine oxidases (PCO) (Weits *et al.*, 2014; White *et al.*, 2017). Oxidized cysteine acts as a secondary destabilizing residue, enabling recruitment of enzymatic activities that lead to protein arginylation and degradation (Dissmeyer, 2019; Holdsworth *et al.*, 2019). For example, this pathway was shown to regulate the abundance of methionine-cysteine (MC)-initiating ERF-VII proteins to induce metabolic adaptation to hypoxia (Gibbs *et al.*, 2011; Licausi *et al.*, 2011). Therefore, meristem proteins equipped with the MC-degron hold potential to act as oxygen-dependent developmental regulators in these tissues. Indeed, oxygen-dependent proteolysis of MC-initiating VERNALISATION 2 (VRN2), LITTLE ZIPPER 2 (ZPR2), and ERF-VIIs was lately found to regulate different aspects of plant development (Fig. 1) (Gibbs *et al.*, 2018; Shukla *et al.*, 2019; Weits *et al.*, 2019).

On the shoot side, ZPR2 was shown to regulate the activity of the meristem to initiate new leaves. Previously, ZPR proteins were shown to operate as post-translational repressors of class III homeodomain leucine zipper (HD-ZIP III) transcription factors involved in several aspects of plant development, including leaf patterning, vasculature development, embryogenesis and root architecture (Ariel *et al.*, 2007; Wenkel *et al.*, 2007; Kim *et al.*, 2008). In the SAM, altered ZPR2 abundance led to changes in the expression of HD-ZIP III target genes involved in SAM activity and meristem size, hinting at those as downstream players of this pathway. Therefore, hypoxia-dependent stabilization of ZPR2 supports a role for molecular oxygen as a developmental cue that regulates shoot meristem activity. Moreover, enhanced stability of the polycomb VRN2 protein at the SAM hints at an additional possibility for oxygen concentrations to affect chromatin dynamics in the shoot apex, including those that control the transition to flowering (Gibbs *et al.*, 2018).

In roots, hypoxia-related transcripts increase in the later stages of LRP development, concomitantly with the establishment of the LR meristem. This has been proposed to be caused by the stabilization of ERF-VII transcription factors upon the establishment of hypoxic conditions. In LRPs, the ERF-VII RAP2.12 interacts with Auxin Response Factor 7 (ARF7) to represses the genes *LBD16*, *LBD18*, *IAA19* and *PUCHI*, thereby ending the LRP developmental program in favour of LR establishment. The involvement of this

class of transcriptional regulators in shaping root architecture was also supported by the observation that a quintuple mutant, almost entirely deprived of ERF-VII activity, showed an increased number of lateral roots, while overexpression of a stabilized RAP2.12 protein led to a reduced density of LRs. Curiously, in the *erf-VII* mutant, LRPs often developed in close proximity, suggesting that hypoxia mediated ERF-VII stabilization is also important for proper root architecture by controlling the timing and spacing of LRP. Taken together with the described function of ERF-VII in apical hook opening (Abbas *et al.*, 2015) and grape bud burst (Meitha *et al.*, 2018), these results extend the role of this TF family as a regulator of anaerobic metabolism to plant development. The impact of oxygen availability on root meristem activity might also extend outside of the ERF-VII domain, since O_2^- and H_2O_2 concentrations were shown to control the stability of PLETHORA 2 (PLT2), a key regulator of RAM size and activity (Yamada *et al.*, 2019). Secreted peptides initiate a signalling cascade that ensure endogenous balance of these reactive oxygen species (ROS), although availability of oxygen itself is also likely to contribute to ROS abundance (Matsuzaki *et al.*, 2010).

Flooding associated with acute hypoxia is known to drive several morphological adaptations that improve oxygen acquisition and delivery. For example, several plant species have the capacity to improve oxygen provision under submergence through aerenchyma formation, shoot elongation and adventitious root development (Voesenek & Bailey-Serres, 2015). While hormones and secondary messengers are well known to play a key role, it is tempting to speculate that the Cys/Arg branch of the N-degron pathway has also been adopted through evolution to take part in these processes. In support of this hypothesis, hypoxic treatments (2% O_2) were shown to lead to root bending by interfering with gravitropic growth, which may act as a mechanism by which to avoid areas of the soil with reduced oxygen availability (Eysholdt-Derzsó & Sauter, 2017). Surprisingly, hypoxia-dependent stabilization of ERF-VII counteracts this process, as *erf-VII* mutants show enhanced root bending, providing a control mechanism on the slanting response. Instead, the process that promotes root bending under hypoxia is linked to asymmetric redistribution of auxin by PIN2 transporters, but how this is achieved remains to be elucidated. The role of ERF-VIIs in flooding-adjusted plant development is also supported by the involvement of the ERF-VII member HRE2, which promotes AR elongation in Arabidopsis under hypoxia (2% O_2) (Eysholdt-Derzsó & Sauter, 2019). In the near future, cell-specific transcriptome analyses applied to tissues subjected to flooding stress components will disentangle the contribution of each factor to the developmental adaptation of plant organs to this environmental challenge.

VI. Evolution of the meristematic low oxygen niche

In animals, a role for oxygen has been extensively demonstrated in a range of developmental processes, including embryogenesis, heart development, angiogenesis and stem cell maintenance (Mohyeldin *et al.*, 2010; Hammarlund *et al.*, 2018). As mentioned for tumour cells, the hypoxia-inducible factors HIF-1 and HIF-2 are essential for the tissue to cope with hypoxia and to maintain stem cell fate (Giaccia *et al.*, 2003; Semenza, 2007; Pietras *et al.*, 2009; Ito & Suda, 2014). In plants, the increasing number of proteins found to be regulated by the Cys/Arg branch of the N-degron pathway broadens the potential that oxygen holds as a developmental regulator in this kingdom. Moreover, the extent of the role that oxygen plays in plant development might be indirectly evaluated by looking at the conservation of the N-degron pathway substrates characterized so far. N-degrons may have arisen during evolution as a result of a mutation of the N-terminally exposed amino acid into a destabilizing residue, or a new start codon may be generated to precede an existing destabilizing amino acid. The latter likely occurred following the duplication of an ancestral polycomb SUPRESSOR OF ZESTE 12 protein (Chen *et al.*, 2009; Gibbs *et al.*, 2018). Here an internal MC sequence was fixed in gymnosperms, which was subsequently followed by N-terminal truncation of the VRN2 protein to expose it for regulation by the Arg/N-degron pathway in angiosperms, linking environmental regulation to epigenetic control of plant development and vernalisation via the polycomb repressive complex 2 (Fig. 3).

The MC N-degron in ZPR2 may have been generated in a similar manner. ZPR2 belongs to a class of microproteins that contain a single functional domain and regulate their ten-times larger HD-ZIP III targets at the post-translational level (Staudt & Wenkel, 2011). Orthologs of HD-ZIP III are present in all land plants and green algae (Floyd *et al.*, 2006). Interestingly, class III HD-ZIP in *Gingko biloba* and the lycophyte *Selaginella moellendorffii* already show expression in the vasculature and SAM, indicating that their role in tissue and organ development was established early on in evolution (Floyd *et al.*, 2006). ZPRs have been proposed to have originated through a duplication event of a *HD-ZIP-III* paralog in a common ancestor of euphyllophytes (Fig. 3). The subsequent loss or degeneration of all of the functional domains, except the leucine zipper (LZ) interaction motif, has led to the evolution of ZPRs in ferns and seed plants (Floyd *et al.*, 2014). In angiosperms, the ZPR2 LZ domain was shown to retain sufficient sequence similarity to form heterodimers or tetramers with its HD-ZIP III targets (Wenkel *et al.*, 2007; Kim *et al.*, 2008; Husbands *et al.*, 2016), but the lack of a functional homeodomain (HD) likely abolishes their ability to bind to DNA. Instead, the sequence preceding the LZ domain in angiosperm ZPRs contains a highly conserved cysteine in second position (Weits *et al.*, 2019), marking them as N-degron pathway substrates. Few ZPR-like sequences were also identified in gymnosperm and monilophytes taxa, albeit their predicted protein sequences do not initiate with MC (Floyd *et al.*, 2014). This could indicate that O_2-sensitive ZPRs are unique to angiosperms, although a more extensive survey should be called for when more gymnosperm and monilophyte sequences become available. Every angiosperm species analysed so far also encodes for a second group of ZPRs which have lost their HD, resulting in a truncated protein composed almost entirely of the LC domain and lacking an MC N-terminus. The two ZPR clades have largely overlapping expression patterns and, on the basis of overexpression and mutant studies, a clear functional separation of the ZPR clades is not obvious (Wenkel *et al.*, 2007; Kim *et al.*, 2008; Xu *et al.*, 2019). Rather, both O_2-dependent and

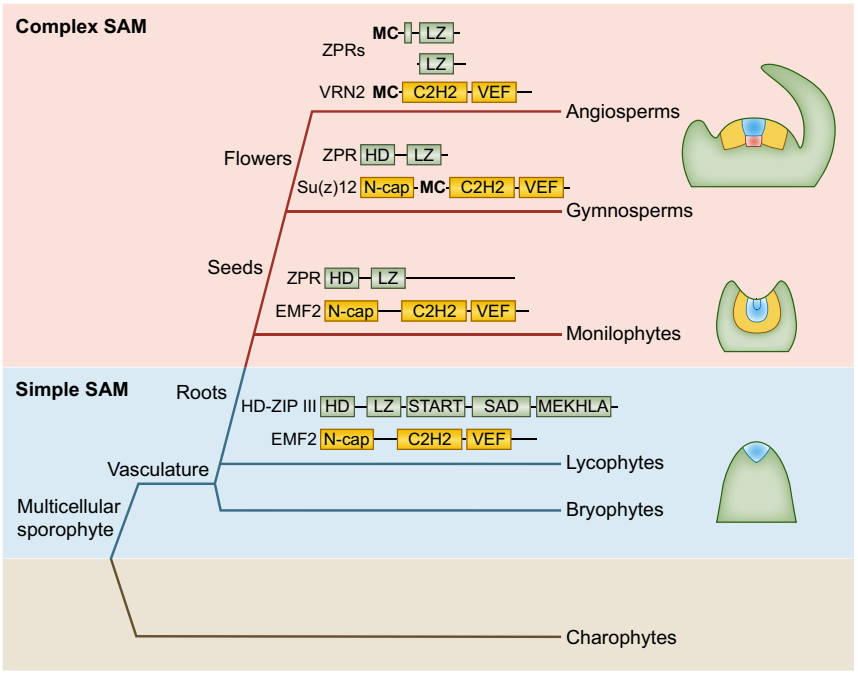

Fig. 3 Schematic overview of the evolution of oxygen-dependent ZPR2 and VRN2 proteins and their correlation to shoot anatomy. Phylogenetic tree representing taxa within the plant kingdom serves as a scaffold to depict the progression of a simple shoot meristem, where all cells originate from one or few apical cells in bryophytes and lycophytes to a complex zonal and multicellular shoot apical meristem (SAM) in ferns and seed plants. The evolution of LITTLE ZIPPER 2 (ZPR2) and VERNALIZATION 2 (VRN2) from ancestral class III homeodomain leucine zipper (HD-ZIP III) and polycomb group protein EMBRYONIC FLOWER 2 (EMF2) progenitors is also shown above each branch, including the acquisition of an N-terminal MC sequence in the protein. Green and yellow boxes represent conserved domains of HD-ZIP III and EMF2, respectively. The known functional domains for each protein are schematically indicated in the figure. For HD-ZIP III and ZPR: leucine zipper (LZ), homeodomain (HD), Steroidogenic Acute Regulatory protein-related lipid Transfer (START), start adjacent domain (SAD) and MEKHLA (the goddess of lighting, water and rain). For EMF2-related proteins: N-terminal cap (N-cap), C2H2 zinc finger domain (C2H2), and a VEF-domain that is contained in *VRN2*, *EMBRYONIC FLOWER2*, and *FERTILIZATION-INDEPENDENT SEED2* genes.

independent ZPRs appear to act concomitantly in leaf patterning and SAM activity.

ZPRs are themselves transcriptional targets of the HD-ZIP III (Wenkel *et al.*, 2007), and thus form a negative feedback loop with their targets. As discussed before, HD-ZIP III have an ancient origin and play a conserved role in the patterning and regulation of plant morphology (Floyd & Bowman, 2007). It has been proposed that the evolution of ZPRs in vascular plants provided a novel way to regulate HD-ZIP IIIs that led to the generation of more complex morphological structures (Floyd & Bowman, 2007; Floyd *et al.*, 2014). For example, the meristems of extant species of bryophytes and lycophytes comprise one or a few stem cells, whereas the shoot and root apical meristems of seed plants are composed of functionally separable layers and zones (Banks, 2015; Plackett *et al.*, 2015) (Fig. 3). The observation that oxygen-dependent ZPRs and VRN2 appeared in angiosperms raises the question of whether meristem complexity coincides with a role for (low) oxygen in SAM activity and floral transition. Alternatively, chronic hypoxia in meristems may be a conserved feature in land plants, and it is conceivable that this has later been adopted by the Cys/Arg branch of the N-degron pathway to regulate proteolysis of substrates involved in plant development.

Emergence of lateral roots from primordia that originate from pericycle cells is typical of spermatophytes, whereas lycophytes achieve root branching through apical bifurcation. By contrast, mosses only generate simple substrate-anchoring structures, called rhizoids, during their gametophyte phase (Prigge & Bezanilla, 2010; Augstein & Carlsbecker, 2018). From this viewpoint, the evolution of lateral roots and shoot apices clearly predates that of floral organs in angiosperms, the only taxa in which chronic hypoxia in meristems has been characterized so far. Therefore, to understand if chronic hypoxia is a conserved trait of meristems, oxygen measurements should be carried out in fern and angiosperm LRPs, during bifurcation of roots in lycophytes, in shoot apices of gymnosperms and monilophytes, and in the apical cells of bryophytes and lycophytes. This type of measurement represents an exciting but technically challenging endeavour.

VII. Dynamics of oxygen during plant development and interplay with other factors

Based on the recent advances presented in Fig. 1, oxygen may now be placed among other previously identified environmental and metabolic cues that affect plant development. Among these, light and carbon availability (sugars) were shown to act on meristem activation at the postembryonic stages of development. In the root meristem, glucose signalling through target of rapamycin (TOR) is sufficient to activate cell proliferation, while shoot apices requires both light and sugar stimuli to generate new organs (Pfeiffer *et al.*, 2016; Li *et al.*, 2017).

Components of the N-degron pathway have also been shown to be important for light-mediated development (photomorphogenesis) during seedling establishment in Arabidopsis (Abbas *et al.*, 2015). Here, ERF-VII inhibits the opening of the apical hook, which is instead promoted by light. It is possible that this is again achieved by the antagonistic relationship between ERF-VII and ARFs transcription factors, since the ERF-VII RAP2.3 was shown to interact with Auxin Response Factor 6 (ARF6) to inhibit cell elongation (Liu *et al.*, 2018). This ensures that the apical hook remains closed to protect the meristem when the oxygen availability in the soil is low (e.g. during waterlogging), since this stabilizes ERF-VIIs. The interplay between light and oxygen as cues to regulate plant development is intriguing. Previous reports (Abbas *et al.*, 2015; Pfeiffer *et al.*, 2016; Li *et al.*, 2017; Shukla *et al.*, 2019; Weits *et al.*, 2019) studied light and oxygen as two interdependent components of seedling establishment and SAM development. However, it is expected that in green tissues photosynthetic activity also acts indirectly on the Arg/N-degron pathway by providing oxygen as a co-substrate for PCOs to promote the proteolysis of ERF-VII, VRN2 and ZPR2 (Considine, 2018).

Although not experimentally tested, it has also been proposed that ERF-VII may act on the TARGET OF RAPAMYCIN (TOR) pathway through the upregulation of a hypoxia Responsive Universal Stress Protein 1 (HRU1) (Gonzali *et al.*, 2015; Considine, 2018). HRU1 acts to modulate ROS homeostasis under anoxic conditions and induces the GTPase ROP2 (Gonzali *et al.*, 2015), which was shown to inactivate TOR in response to auxin (Schepetilnikov *et al.*, 2017). Thus, since TOR progresses the cell cycle, chronic hypoxia in meristems or callus may be linked to proliferation via ERF-VII stabilization. At the same time, the position of TOR in this pathway allows glucose concentrations to influence cell division rates.

Finally, the occurrence of hypoxic conditions was hypothesized to occur at the quiescent centre (QC) of the RAM, to regulate the balance between proliferation and quiescence (Considine *et al.*, 2017) (Fig. 1), which is essential to prevent consumption of the stem cells. Low oxygen concentrations in the QC could affect the redox potential and thus the production of reactive oxygen (ROS) and nitrogen species (RNS), whose abundance is known to regulate stem cell fate (Tsukagoshi *et al.*, 2010). Additionally, oxygen availability could act via stabilization of substrates of the Cys/Arg branch of the N-degron pathways, such as ERF-VIIs, to regulate the QC. As discussed before, experimental validation of oxygen gradients in the RAM is required to explore this hypothesis further.

VIII. Breakthroughs in oxygen biosensors

Precise mapping of the oxygen concentration in meristems at high spatial and temporal resolutions is an exciting prospective challenge. Currently, two main technologies are typically employed to profile oxygen concentrations in living tissues: Clark-electrodes

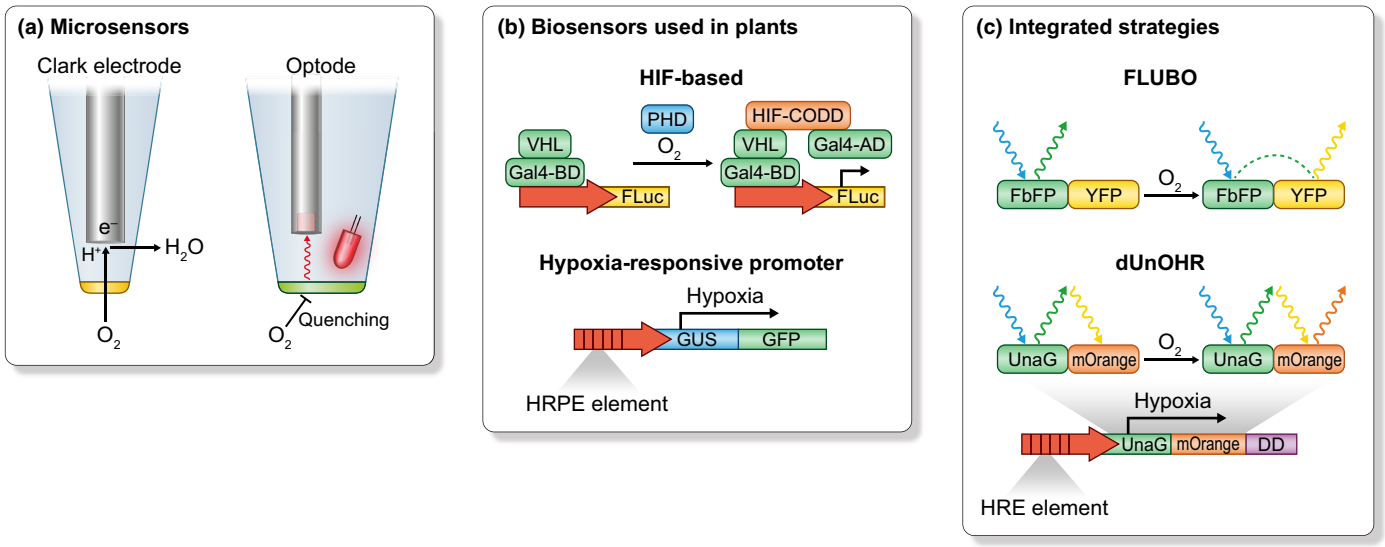

Fig. 4 Strategies for *in vivo* oxygen measurements. (a) Clark electrodes and phosphorescent optodes rely on electron transfer to oxygen and oxygen-dependent quenching of phosphorescence, respectively. (b) Genetically encoded input (upper panel) and output (lower panel) reporters that have been expressed in plants. A heterologous HYPOXIA-INDUCIBLE FACTOR (HIF)-based sensor relies on oxygen-dependent reconstitution of a transcriptional unit in the presence of PROLYL-HYDROXYLASE (PHD), to drive expression of firefly luciferase (Fluc). Here, the C-terminal oxygen-dependent degradation (CODD) domain of HIF-1α was fused to a Gal4 activation domain (AD), which interacts with VON HIPPEL-LINDAU TUMOUR SUPPRESSOR (VHL) in an O_2-dependent manner. The synthetic hypoxia-responsive promoter element (HRPE) promoter consists of several repeats of the DNA enhancer bound by group VII Ethylene Response Factor (ERF-VII) transcription factors and drives expression of reporter proteins GFP and β-glucuronidase (GUS) in hypoxia. (c) Oxygen biosensors based on integrated strategies and potentially applicable to plant studies. The Flavin-binding fluorescent protein (FbFP)-based sensor FLUBO (FLUorescent protein-based Biosensor for Oxygen) is based on oxygen-dependent YFP emission via Förster Resonance Energy Transfer (FRET). The dUnOHR (UnaG mOrange Hypoxia Reoxygenation) reporter integrates hypoxia-dependent inducibility by a synthetic hypoxia responsive element (HRE), oxygen-dependent maturation of mOrange and protein instability conferred by degradation domains (DD).

and phosphorescence-based probes (Fig. 4a). While the first exploits electrolytic reduction of oxygen molecules in an environment sealed by an oxygen permselective membrane, the second relies on oxygen-dependent quenching of phosphorescence (Ast & Draaijer, 2014). Oxygen measurements using miniaturized Clark-type microsensors remain state of the art and were successfully used to obtain oxygen profiles in the shoot apical meristem, although their application is not without downsides. Indeed, the invasiveness of needle-type probes limits continuous time-resolved measurements and the unidirectional insertion into the tissue hinders the generation of three-dimensional reconstructions of the oxygen distribution. Likewise, nanoparticle-based phosphorescent sensors are equally limited by uneven distribution (Avellan *et al.*, 2017) and possible interference with biological reactions, including those involving molecular oxygen. Thus, endogenously encoded biosensors represent a valuable alternative to obtain information about oxygen concentrations in plant tissues.

A genetically encoded hypoxia signalling reporter based on a five-time repeat of the hypoxia responsive promoter element (HRPE, Gasch *et al.*, 2015; Fig. 4b) has been recently used to image hypoxia in the SAM, while the expression of hypoxia-inducible PCO genes was also revealed as a reliable marker of underlying hypoxic conditions (Weits *et al.*, 2019; Shukla *et al.*, 2019). However, these reporters are a read-out of hypoxia signalling and do not report the actual oxygen concentration in the tissue. Their output may also be affected by other factors that impact on ERF-VII stability, such as NO, overall cell energy status and ethylene (Gibbs *et al.*, 2015; Schmidt *et al.*, 2018; Hartman *et al.*, 2019).

Heterologous expression of a mammalian hypoxia-sensor in plants was recently shown to overcome this limitation (Iacopino *et al.*, 2019) (Fig. 4b). This strategy exploited oxygen-dependent interaction between the α subunit of the HIF1 and the VON HIPPEL-LINDAU TUMOUR SUPPRESSOR (VHL), which is enabled by PROLYL-HYDROXYLASE (PHD), which hydroxylate specific proline residues of HIF1-α (Kaelin & Ratcliffe, 2008). In the synthetic sensor designed by Iacopino *et al.* (2019), fragments of HIF1-α and VHL were genetically fused to the GAL4 activation and DNA binding domains from *Saccharomyces cerevisiae*, respectively. In the presence of a functional PHD protein and oxygen, the effector pair was able to activate transcription of a luciferase gene placed under control of a GAL4 responsive promoter. Unlike a *pADH:luciferase* based reporter, this heterologous sensor provided an O_2-specific and reversible signal in plants (Iacopino *et al.*, 2019).

A number of strategies designed for reporting oxygen concentrations in animal, fungal and prokaryotic cells present potential uses in plants. These include genetically encoded sensors based on an oxygen-dependent change of conformation that attenuates or enhances Förster Resonance Energy Transfer (FRET) between pairs of fluorescent proteins (Youssef *et al.*, 2016). Other proposed sensors are based on the pairing of fluorescent proteins (FP) with differential requirements for oxygen for fluorophore maturation (Potzkei *et al.*, 2012; Erapaneedi *et al.*, 2016). Both strategies present the advantage of being ratiometric and therefore deliver information independently of the absolute sensor concentration in the cell.

Moreover, integrated strategies have been successfully used to report oxygen concentrations in bacteria and tumour hypoxia in animals (Fig. 4c). For example, the fluorescent pair UnaG (oxygen independent) and mOrange (oxygen-dependent) was co-translationally fused together with oxygen-dependent and constitutive degradation domains and expressed under the control of a synthetic hypoxia-inducible promoter. Application of this set of sensors, named dUnOHR (dUnaG and mOrange based Hypoxia and Reoxygenation reporter), allowed differentiation between hypoxic and re-oxygenated epithelial cells. In an alternative approach, the Yellow FP (oxygen dependent) and Flavin-binding FP (FbFP; oxygen independent) were genetically linked to produce yellow fluorescence by FRET in the presence of oxygen in bacteria. This strategy has been named as FLUBO (FLUorescent protein-based Biosensor for Oxygen). However, both systems present two critical downsides: first, O_2-dependent chromophore maturation is irreversible, and these sensors therefore exhibit a rather slow response due to their reliance on *de novo* protein synthesis. Secondly, UnaG and FbFP require bilirubin and Flavin mononucleotide as prosthetic groups for fluorescence, respectively (Potzkei *et al.*, 2012; Kumagai *et al.*, 2013). Consequently, they may return faulty outputs when these cofactors are limiting. Moreover, exploitation of the UnaG fluorescent protein in plant species would require genetic engineering of bilirubin biosynthesis to obtain sufficient and homogenous concentrations. The effect of the expression of significant amounts of oxygen binding biosensors in living plant cells should also be considered, especially since these may compete in reactions that require oxygen, or lower the overall oxygen availability in the tissue. Nonetheless, these pioneering endeavours laid the foundations for integrated approaches to generate reliable oxygen reporters for eukaryotic organisms, including plants.

IX. Conclusions

While oxygen dynamics have long been assigned a role in stem cell identity and proliferation in animal biology, a similar signalling function of oxygen to direct development has only recently been studied in plants. In this kingdom, evolution seems to have recruited the Cys/Arg branch of the N-degron pathway to integrate variable oxygen availability into developmental processes. From this perspective, oxygen availability is transduced by three different classes of MC-initiating N-degron pathway substrates: ERF-VIIs, ZPR2 and VRN2. Based on current evidence, VRN2 and ZPR2 appear to act within more specific pathways in shoot meristem activity and reproductive transitions, while ERF-VII act more broadly in the adaptation to tissue hypoxia but also participate in seedling establishment and root architecture. The discovery that meristem niches of the LRP and shoot apex are maintained under hypoxia raises several intriguing questions. First, it is unclear how hypoxia is maintained within these niches. Here, hypoxia may be establishment via respiratory oxygen consumption or sustained by a yet to be discovered oxygen diffusion barrier. Consequently, the catabolic pathways that lead to energy production in meristems require further investigation. A flux of oxygen over a steep oxygen gradient to the meristem would maintain ATP production via oxidative phosphorylation, while

anaerobic metabolism could protect the meristem cells from oxidative threats. Finally, the origin and evolution of hypoxic niches remains enigmatic. Did these co-evolve with oxygen-dependent VRN2 and ZPR2 in angiosperms, or was this feature already present in more ancient plants and later adopted to direct plant development by the Cys/Arg branch of the N-degron pathway. Curiously, stem cells occupy a hypoxic niche in both animals and plants, which hints at convergent evolution of the low oxygen stem cell niche and suggests that it is a critical requirement of complex multicellularity.

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ORCID

Francesco Licausi https://orcid.org/0000-0003-4769-441X
Joost T. van Dongen https://orcid.org/0000-0001-7944-9289
Daan A. Weits https://orcid.org/0000-0003-4423-5568

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See also the Editorial on this article by Sasidharan *et al.*, **229**: 5–7.