Oxygen Sensing and Signaling

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Annu. Rev. Plant Biol. 2015. 66:345–67

First published online as a Review in Advance on January 12, 2015

The *Annual Review of Plant Biology* is online at plant.annualreviews.org

This article's doi: 10.1146/annurev-arplant-043014-114813

Keywords

hypoxia, oxygen, metabolic regulation, fermentation, N-end rule pathway, ERF transcription factors

Abstract

Oxygen is an indispensable substrate for many biochemical reactions in plants, including energy metabolism (respiration). Despite its importance, plants lack an active transport mechanism to distribute oxygen to all cells. Therefore, steep oxygen gradients occur within most plant tissues, which can be exacerbated by environmental perturbations that further reduce oxygen availability. Plants possess various responses to cope with spatial and temporal variations in oxygen availability, many of which involve metabolic adaptations to deal with energy crises induced by low oxygen. Responses are induced gradually when oxygen concentrations decrease and are rapidly reversed upon reoxygenation. A direct effect of the oxygen level can be observed in the stability, and thus activity, of various transcription factors that control the expression of hypoxia-induced genes. Additional signaling pathways are activated by the impact of oxygen deficiency on mitochondrial and chloroplast functioning. Here, we describe the molecular components of the oxygen-sensing pathway.

Contents

INTRODUCTION

Molecular oxygen is involved in a wide variety of biochemical reactions in plants. In photosynthesis, oxygen is produced from the photolysis of water by the photosystems in the thylakoid membranes of the chloroplasts, thereby converting light energy into chemical energy in the form of ATP. Oxygenic photosynthesis is by far the main source of molecular oxygen on Earth (35), and the availability of oxygen in the atmosphere correlates directly with the evolution of photosynthetic organisms, which began approximately 2.3 billion years ago (76). When cellular life first evolved, the concentration of molecular oxygen in the atmosphere was approximately 1,000 times less than it is today. Therefore, one could argue that cells today are nearly always in a high-oxygen environment, and thus have evolved to live at the present ambient oxygen partial pressure (55, 80). Nevertheless, poor oxygen availability to cells can lead to a local energy crisis within the tissue (105) because oxygen is a main substrate of respiratory energy metabolism in aerobic organisms, acting as the terminal electron acceptor of the mitochondrial electron transport chain. In addition to its central role in energy metabolism, molecular oxygen serves as a substrate in many other reactions of plant metabolism, such as fatty acid desaturation and the synthesis of phytohormones such as ethylene, abscisic acid, and gibberellins.

Reactive oxygen species (ROS): oxygen molecules with an unpaired electron that renders them extremely reactive

Oxidative phosphorylation: a metabolic pathway involving the mitochondrial electron transport chain through which ATP is released

The production of reactive oxygen species (ROS) in plant cells is another chemical reaction in which molecular oxygen is involved as substrate (14). High ROS levels can lead to detrimental effects such as damage to lipids, protein, and DNA (1). On the other hand, ROS molecules can also act as important signaling molecules in plant cells (35) and as such are involved in the control of cell differentiation and development (114). In maize anther development, the cellular oxygen concentration controls the fate of the male germinal cell identity by affecting the ROS production within the cells (58). A cell-specific gene expression analysis showed that male germinal cells provoke hypoxia by activating alternative respiratory pathways that produce only a small amount of ATP per oxygen molecule as compared with oxidative phosphorylation. (For a brief

HYPOXIA AND ANOXIA

The condition of low oxygen availability in plants is difficult to define by numeric values or strict concentrations. The common terminology to describe various oxygen conditions is based primarily on a comparison with the present atmospheric oxygen level, which is 21% (v/v), a level referred to as normoxia. Environmental oxygen concentrations higher and lower than this are called superoxia and hypoxia, respectively. Unfortunately, the interpretation of the word hypoxia is often complicated because it is sometimes used to refer to the oxygen concentration in the environment and sometimes to the oxygen concentration inside the tissue, and, for example, plant tissue that is kept in a normoxic environment can be hypoxic inside. Therefore, it is recommended that investigators clearly define the situation (or oxygen concentration) to which the hypoxic situation is being compared.

Anoxia is the situation in which no free oxygen is available. This term is also often used to describe oxygen concentrations inside tissue that are very close to zero. However, such a situation does not automatically imply that no oxygen is available for (bio)chemical reactions, because steep gradients of oxygen provide a strong diffusion potential to drive a flux of oxygen from high to low concentrations. It is therefore recommended that investigators use the term severe hypoxia to describe this situation instead.

discussion of the terminology used to describe oxygen availability, see sidebar Hypoxia and Anoxia.) To prevent energy dissipation, the oxygen consumption rises, which concomitantly leads to low oxygen concentrations—and thus low ROS production rates—in the dividing cells (59).

Steep oxygen gradients inside tissues are not exceptional but rather are a common phenomenon in plants (**Figure 1**). Because plants do not have an active distribution system to transport oxygen, the supply of oxygen to tissues depends on passive transport such as diffusion and convection (2, 45). Depending on the length of the transport pathway, the conductivity for gas transport of the tissue, and the oxygen consumption rate of the cells, the plant internal oxygen concentration can drop to values close to zero. In the centers of developing seeds such as those of pea, maize, sunflower, and bean, oxygen concentrations of less than 5 kPa have been measured (16). Interestingly, the oxygen concentration significantly increased in green seeds during the day owing to the photosynthetic activity of the cotyledons. Steep oxygen concentrations have also been found in stem tissue: Although the oxygen concentration of the epidermal cells was in equilibrium with the surrounding air, the oxygen concentration in and around the vascular bundles was only approximately 20 kPa. Subambient oxygen concentrations have also been observed in bulky storage tissues such as potato tubers (68), large fruits (10, 45), and roots (4) (**Figure 1**).

Areas of low oxygen concentration inside plant tissues expand when the availability of oxygen from the environment decreases, e.g., during waterlogging of the soil after a period of heavy rain (61). Such events, which can happen regularly during a plant's life, might even lead to a temporary absence of oxygen inside parts of the plant. In extreme cases, when plants are submerged, the stress impact induced by the lack of oxygen from the air is also combined with the effect that the water around the plant has on the light quantity and quality as well as on the CO_2 availability that is required for photosynthesis (91, 132). Owing to this composite nature of flooding stress, a wide variety of responses exist to allow either stress avoidance or true tolerance; these have been reviewed extensively (7, 125).

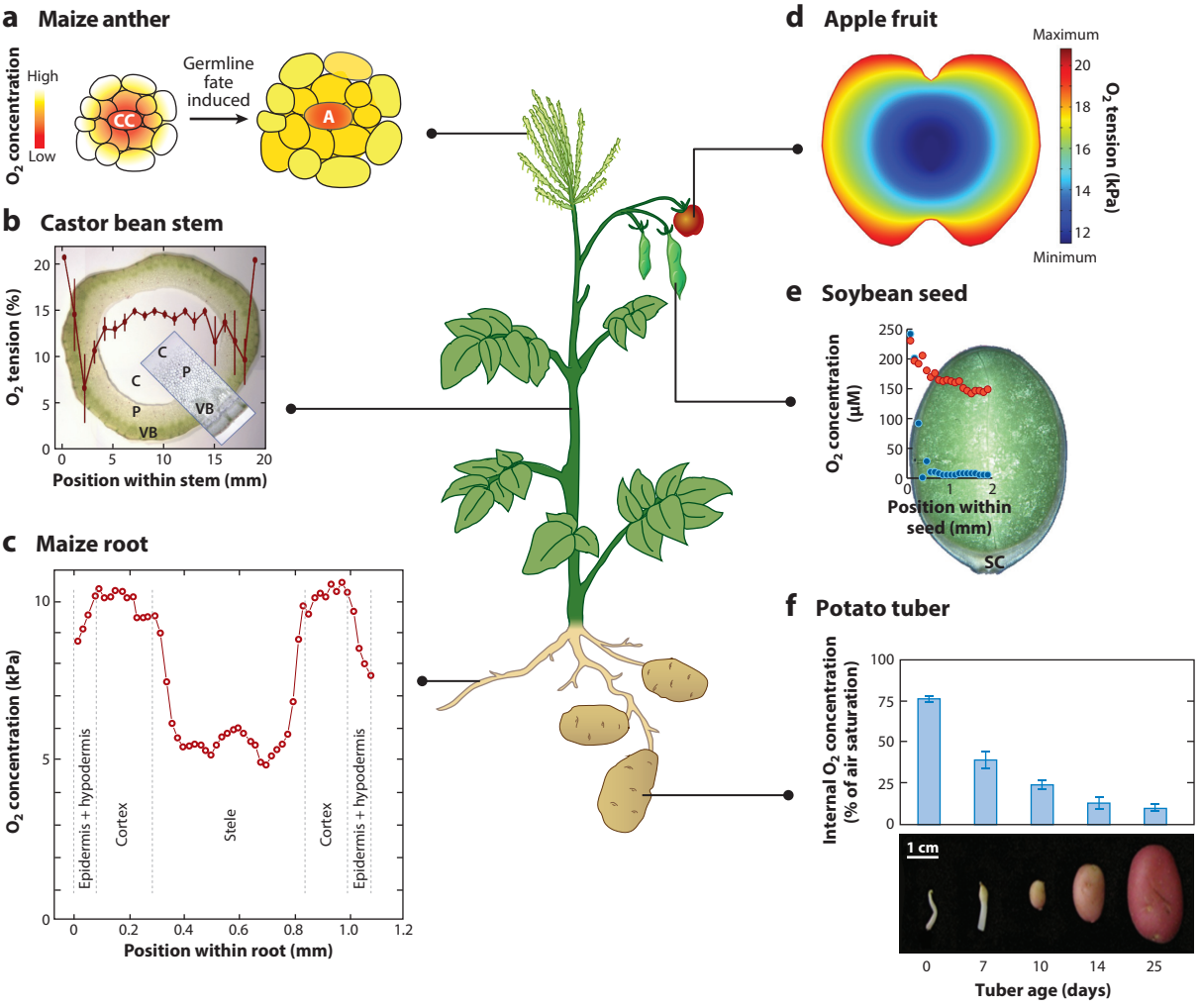
In this review, we describe the gradual and reversible responses of plant tissue that is exposed to decreasing oxygen availability. We also discuss current knowledge about the sensing and signaling pathway for oxygen in plants and how this is involved in regulating the reversible responses to low oxygen.

REVERSIBLE RESPONSES TO HYPOXIA

Energy Metabolism and Fermentation in Low-Oxygen Conditions

The primary impact of reduced oxygen concentration inside plant tissues is on the metabolic pathways in which oxygen acts as a substrate, such as respiratory energy metabolism. Secondary responses that are induced by low oxygen in plants, such as aerenchyma formation (111) or rapid stem elongation (126) to reach the water surface, aim to relieve the hypoxic stress by increasing permeability to oxygen. Whereas most morphological adaptations are irreversible, the metabolic responses to low-oxygen stress can be rapidly reversed as soon as the oxygen availability increases again. This, of course, requires appropriate regulation mechanisms.

When the oxygen concentration drops to a level at which oxygen becomes limiting as a substrate for cytochrome *c* oxidase, ATP production via oxidative phosphorylation is no longer able to meet cellular demands for ATP. This can be compensated for by the activation of glycolytic activity to increase the ATP production by substrate-level phosphorylation (33). However, compared



with oxidative phosphorylation's net theoretical maximum production of about 36 ATP units per hexose unit, the ATP yield per glucose unit that can be derived through glycolysis alone is much lower: only 2 ATP units. Therefore, glycolytic activity must be strongly upregulated under hypoxic conditions to generate sufficient ATP. This phenomenon, also known as the Pasteur effect (after Louis Pasteur, who discovered it in 1857), requires the efficient recycling of NAD^+ from NADH; otherwise, glycolysis will become limited by the availability of NAD^+. Therefore, the ethanol and lactate fermentation pathways are induced by activating the expression of the enzymes pyruvate decarboxylase and alcohol dehydrogenase (for the ethanol pathway) and lactate dehydrogenase (for the lactate pathway), both of which use pyruvate and NADH as substrates.

An alternative pathway to recycle NAD^+ from NADH under low-oxygen conditions is via a futile nitric oxide (NO) cycle: Under hypoxic conditions, nitrite reduction by nitrate reductase increases, leading to NO production (72). NO is oxidized to nitrate again by class-1 nonsymbiotic hemoglobins that are specifically expressed when the oxygen concentration is low (24, 50). This cycle requires NADH as a cosubstrate and plays a role in balancing the antioxidant status of the cytosol (53). Indeed, lactate accumulation decreased in plants that produce large amounts of NO upon hypoxia (87, 88), supporting the hypothesis that the futile NO cycle alleviates fermentation.

Although enzymes of fermentative metabolism have been found in all plant species investigated so far, not all species tolerate a hypoxia-induced energy crisis equally well. Differences in metabolic tolerance to hypoxia arise from variations in the ability to control the carbohydrate consumption rate during anoxia. Comparison of two rice varieties differing in anoxia tolerance suggested that fermentative activity is controlled by mutual regulation of the glycolytic enzymes phosphofructokinase (PFK) and pyrophosphate:fructose-6-phosphate-1-phophotransferase (PFP) in an anoxia-tolerant variety, whereas an intolerant variety showed no correlation between PFK activity and fermentation rate (34). An extensive analysis of PFK and PFP gene expression and enzyme activities suggested that coordinated expression of specific isoforms with different catalytic properties may be involved in controlling the glycolytic activity during low-oxygen stress (82).

Glycolysis and the TCA cycle: the catabolism of sugars to generate redox equivalents used by the oxidative phosphorylation pathway to produce ATP in aerobic organisms

Fermentation: metabolic pathways that produce ATP when too little molecular oxygen is available for oxidative phosphorylation

Figure 1

Variations in oxygen concentration inside plants both between tissues and through time. (*a*) Oxygen concentration in maize anthers. Hypoxia is thought to be actively induced within the tissue by the activation of alternative respiration pathways [alternative oxidases (AOXs)] that use molecular oxygen to dissipate redox energy as heat rather than to convert it into ATP. This decreases the redox potential and reactive oxygen species production, and is followed by the differentiation of central cells (CC) into archespores (A). Modified from Reference 131 with permission from AAAS. (*b*) Oxygen profile as measured using a microsensor through a castor bean (*Ricinus communis*) stem. The hollow center (C) of the stem acts as a lateral transport tube for gas transport through the stem, while the parenchyma cells (P) of the cortex, and especially the highly metabolic active cells of the vascular bundle (VB), are exposed to hypoxic conditions. Modified from Reference 117 with permission from the American Society of Plant Biologists. (*c*) Radial oxygen profile across the primary root of maize. Note that the oxygen concentration is higher in the cortex than in the surrounding tissues of the rhizodermis and the stele, which results from lateral oxygen transport from the shoot toward root tissues. Modified from Reference 4 with permission from Oxford University Press. (*d*) Model of the oxygen partial pressure distribution along the vertical axis of an apple fruit. Gas transport through bulky fruit tissue is mediated mainly via an extensive network of gas-filled apoplasmic spaces between the cells. The color bar indicates oxygen partial pressure (in kPa). Modified from Reference 46 with permission from Oxford University Press. (*e*) Oxygen distribution map of soybean seeds measured under light (*red circles*) or dark (*blue circles*) conditions. The very poor oxygen availability results from the densely packed cell structure of the embryo and its surrounding seed coat (SC). In green seeds, however, this is compensated for by the photolysis of water by photosystem II in the cotyledons within the developing seed. Modified from Reference 16; copyright © 2009 John Wiley and Sons. (*f*) Internal oxygen levels in the cores of tubers at different developmental stages. The oxygen concentration decreases as tuber size increases, thereby inducing various metabolic responses to low-oxygen stress. Modified from Reference 68 with permission from Oxford University Press.

Reversible Metabolic Responses to Save Energy During Hypoxia

Because the efficiency of ATP production by substrate-level phosphorylation and concomitant fermentation is much lower than ATP production via oxidative phosphorylation by the mitochondrial electron transport chain, the use of ATP by primary and storage metabolism decreases when the oxygen availability declines (29). Various adaptive metabolic responses can help to save ATP, and combinations of these responses likely occur simultaneously in hypoxic cells. The efficacy of such a strategy is highlighted by the flooding tolerance displayed by rice varieties that possess the *Sub1A* gene, which controls metabolic quiescence following submergence. In addition, it is unlikely that the metabolic responses described are switched on only below a specific threshold level of oxygen; more likely, a gradual activation occurs following changes in the oxygen level through time or along a gradient.

However, research to determine the correlation between pathway activity and oxygen concentration requires methods to determine (sub)cellular oxygen concentration in a nondestructive way. Progress is being made in obtaining a protein-based oxygen reporter [fluorescent protein–based biosensor for oxygen (FluBO)] that can be expressed by cells and changes its fluorescence properties depending on the oxygen concentration at the time that the fluorophore matures (93). Unfortunately, this reporter system does not allow investigation of dynamic changes in tissue, and further technical developments are required.

Examples of metabolic regulation that occurs in plants to save ATP during hypoxic stress include the following:

1. The activity of storage metabolism is reduced when the oxygen availability decreases (29). The synthesis of lipids and proteins is particularly affected (116, 124) because these pathways require much more ATP than, e.g., the synthesis of starch. As a consequence of reduced storage metabolism, phloem import of photoassimilates toward hypoxic sink tissue also decreases (116). Transport phloem is especially prone to hypoxia in general because it is embedded deeply inside other heterotrophic tissues. In combination with its high ATP demands to energize sucrose retrieval, the phloem is especially vulnerable to changes in energy availability (117). Therefore, sucrose-proton symport–mediated sucrose retrieval in the phloem does not depend entirely on respiratory ATP to balance the electrochemical component of the membrane potential; the potassium channel AKT2 in the phloem is also involved in maintaining the membrane potential of the phloem (27). This strongly reduces the dependence on oxygen for the energization of sucrose retrieval in the phloem.
2. After the onset of hypoxia, the enzymatic breakdown of sucrose occurs preferentially by sucrose synthase rather than by invertase, because the phosphorylation of the hexoses after hydrolysis of sucrose by invertase requires two ATPs as compared with one UTP and one inorganic pyrophosphate when sucrose is split by sucrose synthase (15). Indeed, hypoxia induces the expression of sucrose synthase genes (11) and upregulates reactions that produce inorganic pyrophosphate in low-oxygen conditions (51). Furthermore, the importance of sucrose synthase activity during hypoxia is underscored by the observation that antisense suppression of sucrose synthase leads to reduced hypoxia tolerance in cucumber (128).
3. Another response of primary metabolism in plants to hypoxia is the bifurcation of the tricarboxylic acid (TCA) cycle. Under nonstress conditions, the gross flux of carbon through the TCA cycle follows the oxidative cycle, which allows maximum production of NAD(P)H. However, under hypoxic conditions, the cycle can (partly) split into an oxidative and a reductive branch (99, 110, 121). The shift between these two flux modes is thought to occur smoothly (38). Theoretically, this way of controlling the TCA cycle activity can regulate the ratio between $NAD(P)^+$ and NAD(P)H in the mitochondria; by doing so, it

would also control the activity of respiratory oxygen consumption by the mitochondrial electron transport chain. However, this remains to be proven to occur during hypoxia. The molecular mechanism controlling the main direction of carbon flux through the TCA cycle also remains to be clarified.

4. The activity of the mitochondrial electron transport pathway changes with the oxygen concentration as well (39). When the oxygen concentration around root tissues decreases, the rate of respiratory oxygen consumption also decreases. The oxygen response curve of respiratory activity of roots actually shows a biphasic behavior. In a study by Zabalza et al. (137), the oxygen consumption rate declined steadily but slowly when the oxygen concentrations around the tissue decreased from 21% (air saturation) to approximately 4%. However, below 4% oxygen, the rate of oxygen consumption dropped steeply.

Supercomplex: a single large structure assembled from multiple protein complexes

Two explanations for the biphasic behavior mentioned in item 4 are currently being discussed in the literature. One hypothesis is based on a mathematical modeling approach using Michaelis-Menten enzyme kinetics, and predicts that the cellular oxygen concentration in the core of the tissue is limiting for the activity of cytochrome *c* oxidase. When the oxygen concentration around the tissue drops gradually through time, the area of the tissue in which oxygen is a limiting substrate for respiration increases, and the oxygen consumption rate concomitantly decreases (3). The other hypothesis is that proactive regulation of respiratory metabolism keeps control over the rate of respiration to optimize the rate of oxygen consumption to oxygen availability (85).

Convincing evidence about the molecular mechanism of respiratory control as suggested by the second hypothesis is still lacking, although the suggestion that regulation of NAD(P)H production by the TCA cycle is involved in regulating respiratory oxygen consumption is plausible (see item 3 above). In addition, the plant mitochondrial electron transport chain is characterized by a multitude of entry points for electrons (102). For example, in addition to the NADH dehydrogenase (complex I), two forms of type-2 NAD(P)H dehydrogenases exist that are located at either the inner or outer side of the mitochondrial inner membrane. The oxygen use efficiency of respiration (defined as the amount of oxygen used to produce one unit of ATP) differs substantially between the various alternative electron transport pathways, but experimental evidence that the oxygen concentration influences the electron transport pathway is sparse. Supercomplexes between the various respiratory complexes of the mitochondrial electron transport chain dissociate under low-oxygen conditions, which was interpreted to reduce the input of electrons via complex I while preferring the activity of the type-2 dehydrogenases (96). Differential expression of the various alternative components of the electron transport chain is also observed under various stress conditions, including hypoxia (97).

It should be noted, however, that a change in gene expression and concomitant enzyme abundance does not necessarily lead to a change in enzyme activity. Protein modifications are often needed to activate an enzyme. Much progress has recently been made on understanding how oxygen sensing is involved in adapting metabolism to low-oxygen conditions. Nevertheless, a better understanding of the integration between control over gene expression and posttranslational regulation mechanisms that control energy metabolism is still needed.

DIRECT OXYGEN SENSING

The Transcriptional Response to Anaerobiosis in Plants

As mentioned above, hypoxic conditions induce many reversible responses in plant tissues. Both species-specific and universal responses to low oxygen availability in plants involve at least one transcriptional regulation step. However, changes in specific mRNA levels must be evaluated in

Proteolysis: the breakdown of proteins into peptides or amino acids

light of the translational potential of cells that experiences oxygen scarcity. Reduction in respiratory ATP production has been proposed to account for the substantial dissociation of ribosomal subunits and the unloading of multiple ribosomes (polysomes) from mRNAs (6, 17), and restoration of aerobic conditions was sufficient to reverse this dissociation (6). More recently, ribosome profiling analyses indicated that, under hypoxia, translation initiation (rather than elongation and termination) is hampered (56).

At the transcript level, plant adaptive programs to low oxygen include the accumulation of transcripts coding for enzymes involved in metabolic reprogramming (74). At least one isoform of each enzyme responsible for metabolic adaptations to low oxygen, such as pyruvate decarboxylase, alcohol dehydrogenase, lactate dehydrogenase, PFK, and alanine aminotransferase, is induced by treatments that lead to low oxygen in all higher plants tested so far (81). Upregulation of the ethylene biosynthetic genes *ACC synthase* (*ACS*) and *ACC oxidase* (*ACO*) and the jasmonate signaling repressor gene *JASMONATE ZIM DOMAIN PROTEIN 3* (*JAZ3*) provides a link between hypoxia and hormone-directed growth (81). A small number of ROS-responsive transcripts are also upregulated under hypoxic conditions, and an additional set is induced when oxygen levels fall below a minimum concentration that prevents inhibition of the terminal step of the mitochondrial electron transport chain, with ROS thereby actually accumulating (19). In this case, ROS-scavenging enzymes, protein chaperones, and inhibitors of lipid peroxidation are induced to contain the oxidative damage (94). The mitigation of oxidative stress is also required when the plant returns to normoxic conditions, and therefore antioxidant and chaperone proteins play an important role in ensuring survival after temporary submergence.

Many genes belonging to the core anaerobic response code for proteins of unknown function. Recent studies have shed light on the functions of some of them, leading to the identification of negative regulators of the anaerobic response and highlighting the importance of the reversibility and timely control of the anaerobic response (129). In fact, transcript profiling studies revealed that the initial molecular response to hypoxia in *Arabidopsis* is rapidly reversed when oxygen availability is restored to normoxic levels (17, 69). Moreover, the anaerobic response needs to be integrated with the signals coming from the perception of energy and substrate availability and the metabolic demand of each cell (83).

N-End Rule Pathway–Dependent Activation of the Anaerobic Response

Group-VII ethylene response factors (ERFs) exert major control over the metabolic adaptation to hypoxia and thereby contribute to the low-oxygen response (7) (see also sidebar Group VII of the ERF Transcription Factor Family). The first hints of the importance of this class of transcription factors in oxygen perception came with the identification of variety-specific ERF-VII genes responsible for adaptation to or escape from submergence-related anaerobiosis in rice (42, 135). Subsequent studies provided a more in-depth characterization of the ERF-VII members and allowed investigators to dissect the direct control exerted over them by oxygen (25, 31, 69).

In fact, the abundance and concomitant activity of ERF-VII transcription factors are regulated through continuous proteasomal degradation under aerobic conditions. The series of biochemical reactions that ultimately leads to proteolysis is known as the N-end rule pathway, as it depends on the N-terminal amino acid residue that is exposed in a protein or peptide (see sidebar The N-End Rule Pathway). Most of the ERF-VII transcription factors possess an N-terminal domain characterized by a cysteine residue in the penultimate position (MCGGAI) (7) (**Figure 2**). This amino acid sequence provides the molecular signature that is recognized by specific aminopeptidases to remove the initial methionine, thus leaving the cysteine exposed as the N-terminal residue. The cysteine can be oxidized by plant cysteine oxidases (PCOs) using oxygen as a cosubstrate to

GROUP VII OF THE ERF TRANSCRIPTION FACTOR FAMILY

ERF transcription factor proteins have been classified as members of the ERF/AP2 superfamily that contain a single APETALA 2 (AP2) domain (100). Based on a few specific positions in the DNA-binding domain and the presence or absence of additional motifs, the ERF family is further subdivided into 10–12 groups (84). Most of the group-VII ERFs are characterized by the N-terminal consensus, with an oxygen-sensitive cysteine in the penultimate position (MCGGAI) (7). The number of ERF-VII members varies greatly among species, and is usually higher in monocots (70). Based on the occurrence of additional conserved domains apart from the AP2 DNA-binding domain and the conserved N-terminal consensus, the ERF-VII group can be further divided into two classes, which may have diverged early in the evolution of seed plants (119). The group-1 proteins RAP2.2 and RAP2.12 and the group-2 protein RAP2.3 are constitutively and ubiquitously expressed in *Arabidopsis*; the two other ERF-VII members are upregulated by low-oxygen conditions and therefore called hypoxia-responsive ERFs (HRE1 and HRE2) (70). RAP2.2 and RAP2.12 are able to activate expression of anaerobic genes, including *HRE1* and *HRE2*, and their concomitant silencing also attenuates the hypoxic response (44, 69, 89).

generate a sulfinic-acid adduct (129). This moiety in turn acts as an acceptor for the condensation of an arginine residue by arginyltransferases (37). The presence of an exposed N-terminal arginine is recognized by PROTEOLYSIS (PRT) proteins, which are single-subunit E3 ubiquitin ligases that add ubiquitin units to a proximal lysine residue (28). Once polyubiquitinated, the ERF-VII proteins are targeted for degradation via the 26S proteasome. This mechanism provides an efficient way to induce oxygen-dependent molecular responses in a cell: A decrease in oxygen availability hinders oxidation of the Cys2 residue, leading to ERF-VII accumulation in the nucleus.

Ubiquitin: a small regulatory protein that can be attached to another protein substrate as a posttranslational modification

NO is also involved in Cys2 oxidation but is not sufficient to trigger proteolysis by itself (32). Low NO levels in *Arabidopsis* and barley stabilize ERF-VII proteins under aerobic conditions, although concurrent availability of oxygen and NO is required to induce ERF-VII degradation (32). A role for nonsymbiotic hemoglobins (nsHbs) has been proposed to restrict the oxidation of the N-terminal cysteine of RELATED TO AP2 12 (RAP2.12) and therefore promote the anaerobic response (52). Because nsHb1 is itself a direct target of RAP2.12 in *Arabidopsis*, a feed-forward loop would be generated in this way (69). The requirement of NO for the oxidation of N-terminal cysteines by PCOs remains elusive, as these proteins could oxidize free cysteine or cysteine-containing peptides in vitro in the absence of NO donors (129). Two scenarios can be

THE N-END RULE PATHWAY

The process involving covalent modification of N-terminal amino acids, ubiquitin ligation, and proteasomal degradation is defined as the N-end rule pathway. It represents a general mechanism that determines the half-life of many proteins in a cell. N-terminal amino acids that promote proteasome-mediated proteolysis are considered to be N-degrons. They can be divided into primary, secondary, and tertiary destabilizing residues (123). In eukaryotes, including plants, primary amino acids are positively charged amino acids (Arg, His, and Lys) or bulky hydrophobic amino acids (Phe, Tyr, Trp, Ile, and Leu) (112). Primary destabilizing residues are recognized by specialized E3 ubiquitin ligases that add multiple ubiquitin units to proximal Lys residues. Secondary destabilizing amino acids are negatively charged residues (Glu, Asp, and oxidized Cys) to which an Arg residue is conjugated by an arginyl-tRNA transferase (ATE). Tertiary destabilizing residues are subjected to covalent modifications that turn them into secondary destabilizing residues. An example of this is the deamidation of Asn and Gln and the oxidation of Cys.

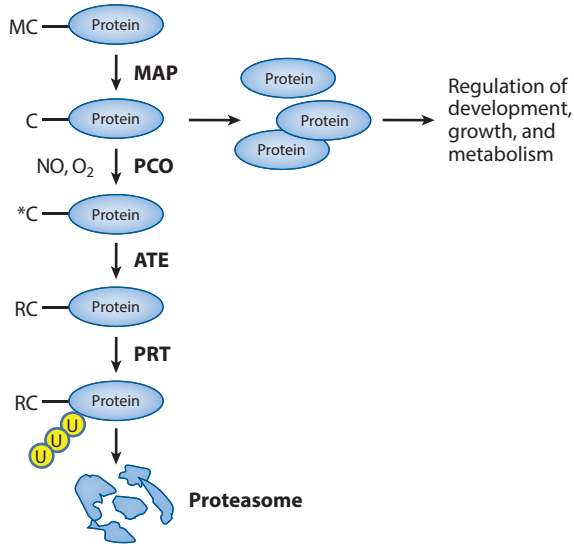


Figure 2

The cysteine branch of the N-end rule pathway in plants. Proteins possessing a cysteine in the second N-terminal position undergo demethionylation via methionine aminopeptidase (MAP), which exposes the cysteine at the N terminus to the activity of plant cysteine oxidases (PCOs). In the presence of oxygen and nitric oxide (NO), PCO oxidizes cysteine to sulfinic or sulfonic acid, which acts as a signal for arginyltransferases (ATEs) to conjugate an arginine residue. The N-terminal arginine is recognized by the PROTEOLYSIS (PRT) E3 ligases, which then polyubiquitinate the protein at proximal lysine residues, thereby causing its degradation by the proteasome complex. The lifetime of ethylene response factor (ERF) transcription factors is determined by this N-end rule pathway, thus controlling the expression of various genes that are involved in the adaptive response of plants to hypoxia in an oxygen concentration–dependent manner. Additional abbreviations: C, cysteine; *C, oxidized cysteine; M, methionine; R, arginine; U, ubiquitin. (See also sidebar The N-End Rule Pathway.)

envisioned: (*a*) In vivo, PCOs might preferentially use nitrosylated cysteine as an electron source, or (*b*) two independent Cys2 oxidation pathways might exist that are mediated by either PCOs or NO. The observation that either decreasing NO levels or knocking out two PCO genes induces the molecular response to low oxygen supports this second hypothesis. An analysis that considers both factors simultaneously would likely determine which scenario is correct, but such an analysis has not yet been performed.

It should be noted that the environmental oxygen level at which this degradation pathway is activated is dynamic and possibly subject to additional environmental control. First, oxygen concentrations at the positions of plant organs vary greatly with diffusion and consumption rates (4, 16, 46, 68); second, some PCO and ERF-VII proteins are themselves induced by hypoxia (70, 129), and therefore their abundances bias cell sensitivity to variations in oxygen concentrations. Moreover, cysteine oxidation could be affected by other molecules linked to oxygen deprivation. For instance, the accumulation of ROS molecules such as hydrogen peroxide and NO at the onset of anaerobiosis could promote cysteine oxidation. In addition, cytosolic acidification, which has been attributed to the inactivation of H^+-ATPase proton pumps, nucleotide triphosphate hydrolysis, and lactate fermentation (54), may potentially affect cysteine oxidation (73). Interestingly, an artificial decrease of cellular pH by acetic acid supplementation to *Arabidopsis* seedlings also led to the induction of a subset of the core anaerobic genes as well as heat shock proteins involved

in disaggregating and refolding denatured proteins (12). From this perspective, the N-end rule pathway functions as a fine-tuning mechanism for responsiveness to hypoxic conditions, and additionally provides a way to rapidly silence the anaerobic response when normoxic conditions are restored.

Not all ERF-VII proteins are substrates of the N-end rule pathway. A few members of this group do not possess the conserved N-terminal MCGGAI consensus, and one of these, the rice ERF Sub1C, is stable in vitro independently of the oxygen availability (31). The elongation repressor Sub1A, which is present in wild rice varieties that are flash-flood tolerant, is also stable in vitro, most likely because of the absence of a proximal lysine residue that could be used as a ubiquitin acceptor by PRT (31).

Because of the N-end rule pathway, transcriptional regulation is likely to play a minor role in determining ERF-VII activity in the cell; nevertheless, many studies have focused on the changes in mRNA levels for ERF-VII genes (43, 44, 70, 104, 136). For example, several studies have indicated that the hypoxia- and flooding-associated hormone ethylene positively regulates *RAP2.2* (44), *RAP2.3* (18), and *HYPOXIA-RESPONSIVE ERF 1* (*HRE1*) (43) in *Arabidopsis*. This increase in ERF-VII transcripts might play an important role in tolerance by building up a reservoir of mRNA that is ready to be translated and stabilized when oxygen levels decrease.

Additional Levels of Posttranscriptional Surveillance on ERF-VII Proteins

Under aerobic conditions, RAP2.12 is present only at the plasma membrane, whereas under hypoxic conditions it accumulates in the nucleus, where it can activate transcription of its target genes (69). The amino acid sequence of RAP2.12 does not contain hydrophobic regions that could span the phospholipid bilayer; however, its ability to associate with the peripheral membrane proteins acyl–coenzyme A (CoA)–binding protein 1 (ACBP1) and ACBP2 can explain its membrane localization (69). ACBP1 and ACBP2 are the only membrane-localized members of a class of proteins with high affinity for long-chain acyl-CoA fatty acid esters. More recently, these ACBPs were shown to also bind phosphatidic acid and phosphatidylcholine (20, 21, 66). However, the observation that substrates of the cysteine branch of the N-end rule pathway in mice are palmitoylated and membrane localized (8) suggests alternative or additional mechanisms for Cys2 proteins like RAP2.12 to dock to the membrane. It should be noted that, because these modifications involve the cysteine residue at the second position, they would also prevent targeting to proteolysis. Independently of how RAP2.12 associates to the plasma membrane, this transcription factor and possibly other ERF-VII proteins could conceivably accumulate as a reservoir of activators able to quickly induce the low-oxygen response when the oxygen availability decreases.

Hypoxic conditions are able to detach RAP2.12 from the membrane and trigger its accumulation in the nucleus (69), although how this mechanism functions remains to be determined. Lipidic second messengers might play a role, as suggested by the molecular function of ACBPs (133). A direct link with oxygen availability could possibly be provided by the requirement of oxygen in several steps of hydroxylation and desaturation. Interestingly, in baker's yeast (*Saccharomyces cerevisiae*), inhibition of sterol biosynthesis in hypoxic conditions is responsible for the activation of the anaerobic response via the transcription factor sterol regulatory element–binding 1 (Sre1). Whether dissociation of RAP2.12 from ACBPs is required to activate the anaerobic response in plants or the two proteins migrate together toward the nucleus remains to be investigated. It will also be interesting to clarify whether interaction is limited to ACBP1, ACBP2, and RAP2.12 or extends to other ACBPs and ERF-VII factors (49). Because other ACBPs are not membrane localized (133), it is possible that they protect ERF-VII from the N-end rule pathway–mediated degradation in other subcellular compartments.

Rabbit reticulocyte extract: a cell-free system for in vitro protein synthesis based on a lysate of immature red blood cells from rabbit

As mentioned above, the ability to rapidly switch the anaerobic response on and off is extremely important to ensure optimal adaptation to changing environments (65, 69). Displacing ERF-VII proteins from the promoters of anaerobic genes and degrading them upon reoxygenation is unlikely to be sufficient to rapidly silence their expression. In *Arabidopsis*, active repression is instead ensured by the association between HYPOXIA RESPONSE ATTENUATOR 1 (HRA1) and RAP2.12 (36). This transcriptional regulator belongs to the trihelix family, which regulates developmental processes and stress responses (95). HRA1 attenuates the induction of fermentative enzymes to prevent rapid depletion of resources via glycolysis. Under submergence conditions, this is extremely important for plant survival (36).

Similarly to PCOs, HRA1 is induced by RAP2.12 stabilization. This stimulates a feedback loop in which RAP2.12 acts as a positive regulator while its targets restrict its function depending on either environmental conditions (e.g., oxygen availability) or organ- or tissue-specific requirements (129).

Other Substrates of the Oxygen-Dependent Branch of the N-End Rule Pathway

The occurrence of a cysteine in the penultimate position of the N terminus of a protein is not exclusive to the ERF-VII proteins (30). However, in both plants and animals, a cysteine residue occurs significantly less frequently in the second position than in other positions of a protein (69). For instance, in *Arabidopsis*, approximately 200 genes code for proteins containing a Cys2. In several cases, this feature is conserved among plant species, suggesting that the presence of cysteine in the N terminus has structural or regulatory functions important for plant fitness (31). For instance, N-terminal cysteine is essential for the catalytic properties of asparagine synthase (118) and is therefore retained in all isoforms of this enzyme. The dependency of protein stability on the occurrence of Cys2 has been tested in only a limited number of candidates (31, 32), but it has emerged clearly that Cys2 is not the sole prerequisite for direct protein degradation; the following residues of the amino acid sequence also likely play a role. This concept is also supported by the observation that the whole N-terminal motif is conserved in most ERF-VII transcription factors (67). In vitro assays using rabbit reticulocyte extracts revealed that at least one non-ERF-VII transcriptional regulator can be stabilized under hypoxic conditions: the flowering regulator VERNALIZATION 2 (VRN2; splice form AT4G16845.1), which possess an N-terminal cysteine (31). Other Cys2 proteins have been previously associated with the regulation of vegetative development and stress responses, and the role of hypoxia in the regulation of these molecular programs deserves further investigation in the near future (13, 106, 130). The occurrence of physiological hypoxic conditions in specific tissues or cell types (such as the phloem and maize germinal cell initials) has been reported, although the generation of genetically encoded oxygen reporters would support the identification of hypoxic niches in plants (59, 117).

THE LOW-OXYGEN-ASSOCIATED SECONDARY SIGNALING PATHWAY

Low-Energy-Associated Low-Oxygen Signaling

Genetic or biochemical alteration of the cysteine branch of the N-end rule pathway in plants could not completely mimic the response to oxygen deprivation. For instance, abolishing the arginyltransferase or N-terminal arginine-dependent ubiquitin ligase activity from *Arabidopsis* seedlings resulted in the upregulation of only half of the 49 core hypoxia-responsive genes (31), and the increase in the changes in mRNA level was relatively modest compared with the effect

of 1% hypoxia. Feedback control can doubtless explain this discrepancy, but it is equally logical to hypothesize that additional mechanisms abet the complete plethora of anaerobic responses. Additionally, most of the core anaerobic genes are not exclusively regulated by hypoxia, suggesting that signaling pathways common to other stresses might be involved under hypoxia as well. In fact, most if not all adverse environmental conditions impair mitochondrial activity to a certain extent (113). On top of this, hypoxic metabolism, which is based primarily on glycolysis, accelerates normal carbohydrate consumption, leading to starvation conditions (26, 92, 120).

In the light of these observations, it is not surprising that the plant orthologs of the conserved energy sensors sucrose nonfermenting 1–related kinase 1 (SnRK1) and target of rapamycin (TOR) have been indicated as key components in the perception and transduction of the energy starvation signal that is also generated under hypoxic conditions (5, 113). In spinach, high AMP:ATP ratios were suggested to cause SnRK1 activation via phosphorylation (109), and the *Arabidopsis* SnRK1 orthologs AKIN10 and AKIN11 activate members of the S1 class of the basic leucine zipper (bZIP) transcription factor family (bZIP1, bZIP11, and bZIP53) under starvation conditions (5). These transcriptional regulators, which are themselves induced by starvation conditions, activate the expression of enzymes involved in protein, structural carbohydrate, and lipid catabolism for use as alternative carbon sources or to tune down dispensable pathways related to active growth and development (23, 57).

Interestingly, SnRK1 is also able to phosphorylate, and hence directly affect, the activity of HMG-CoA synthase (sterol synthesis), sucrose-phosphate synthase (sucrose synthesis), and nitrate reductase (nitrogen assimilation) (77, 78). In rice, the role of SnRK1 has been explored in the frame of starch utilization to fuel glycolysis under flooding conditions (63). Lee et al. (63) proposed that a sugar starvation signal activates calcineurin B–like interacting protein kinase 15 (CIPK15), which initiates a phosphorylation cascade, acting via SnRK1, that ultimately leads to α-amylase induction by the MYB transcription factor MYBS1 (75). Park et al. (90) suggested that the same mechanism is active in sustaining postembryonic development when rice seeds germinate in anoxic conditions.

The function of the TOR protein complex in plants seems to be antagonistic to that of SnRK1s: It stimulates glycolysis and the TCA cycle, amino acid and protein synthesis, and cell wall synthesis while downregulating proteolysis, amino acid catabolism, and autophagy (113). TOR exerts multilevel control over mRNA translation by regulating ribosomal protein synthesis and rRNA gene expression and maintaining translation reinitiation via phosphorylated eukaryotic translation initiation factor 3 subunit H (eIF3h) (98, 101, 134). As discussed above, low-oxygen and low-energy stresses also overlap in inhibiting protein synthesis: Together with OLIGOURIDYLATE-BINDING PROTEIN 1C (UBP1C), mRNAs that are poorly translated associate into stress granules, where they are protected from nucleolysis until translation-favorable conditions return (108).

The Contribution of Organelle-Derived Signals to Low-Oxygen Responses

Low-oxygen conditions affect the physiology and biochemistry of both mitochondria and plastids in plant cells. Recent reports have revealed that signals generated in these organelles contribute to transcriptional reprogramming in the nuclei of hypoxic cells (**Figure 3**). Control of nuclear gene expression is commonly referred to as retrograde, as opposed to the anterograde signaling that orchestrates organelle gene expression through nucleus-derived signals. In plant, animal, and yeast mitochondria, reduction of the oxygen levels to below the K_m for oxygen of cytochrome *c* oxidase inhibits the activity of the mitochondrial electron transport chain and leads to a transient accumulation of ROS (19, 40). In *Arabidopsis* seedlings, this is followed by the activation of mitogen-activated protein kinase 3 (MPK3), MPK4, and MPK6. ROS accumulation and the concomitant activation of the same kinases were also observed after a few minutes of reoxygenation (19). MPKs

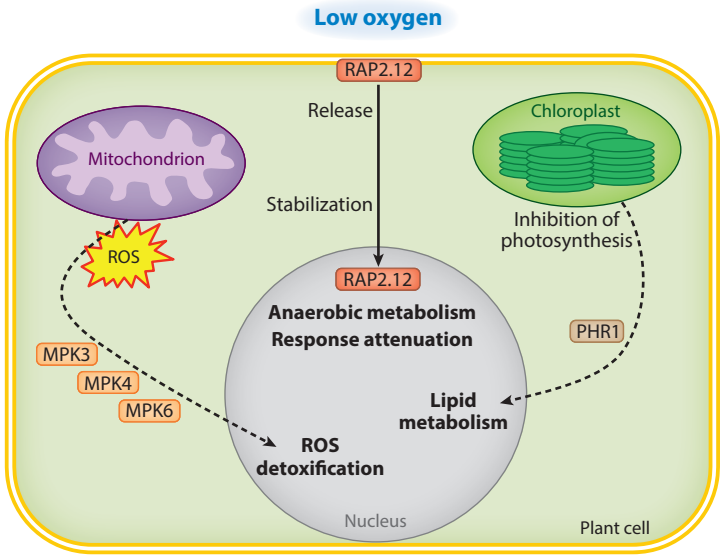


Figure 3

Signaling events contributing to the response to low oxygen in plant cells. Under hypoxic conditions, the plasma membrane–localized transcription factor RAP2.12 is released from the plasma membrane and its degradation is inhibited owing to the low oxygen concentration in the cell. The transcription factor therefore accumulates in the nucleus and simultaneously induces genes involved in anaerobic metabolism and those that attenuate RAP2.12 activity. Furthermore, inhibition of the mitochondrial electron transport chain causes transient reactive oxygen species (ROS) accumulation that acts as a signal, transduced by the mitogen-activated protein kinases MPK3, MPK4, and MPK6, to activate genes involved in ROS scavenging. Moreover, inhibition of photosynthetic activity caused by hypoxia generates a signal of an unknown nature that induces genes involved in maintaining plastidial membrane integrity.

are involved in regulating responses to biotic and abiotic stimuli in plants as well as in other eukaryotes (41). Three classes of kinases (MPKs, MPK kinases, and MPK kinase kinases) regulate each other in a cascade fashion to transmit, amplify, and integrate different signals. Overexpression or inactivation of *MPK6* did not affect the expression of the core hypoxia-response genes under low-oxygen conditions but did alter the levels of transcripts involved in ROS amelioration (19). Pucciariello et al. (94) suggested that, under anoxic conditions, ROS production via membrane-localized NADPH oxidases also activates a specific subset of oxidative stress genes, including different classes of heat shock proteins.

Hypoxia inside the tissue under aerobic environmental conditions also impairs the photosynthetic activity in the chloroplast. However, whether hypoxia occurs under illumination in nature remains questionable, as oxygen production by the photosystems would fuel oxidative phosphorylation. However, it has been observed that, under hypoxia, the concomitant decrease in the oxygen-dependent xanthophyll epoxidation and closure of the stomata that limits CO_2 uptake impair the Calvin-Benson cycle. A similar situation results from prolonged phosphate starvation, a condition that shares some overlap in the transcriptional response to low oxygen (86). Among the genes induced by both low-phosphate and low-oxygen conditions, those coding for galactolipid and sulfolipid biosynthetic enzymes suggested a specific regulation of the chloroplast membranes under hypoxia (60). The induction of these genes depends on a chloroplast-derived signal that posttranslationally activates the MYB transcription factor phosphate starvation response 1 (PHR1) as part of the retrograde signaling pathway.

The Role of MicroRNAs in Hypoxic Signaling

Compared with transcriptome studies on the molecular responses to hypoxia, only a few studies have focused on *trans*-acting small RNAs such as microRNAs (miRNAs) and small interfering RNAs (siRNAs). A first report identified several miRNAs and siRNAs as differentially regulated under near-anoxic conditions (0.1% O_2 v/v in air) (79). The authors suggested that this effect resulted from the inhibition of the mitochondrial electron transport chain, as chemical inhibitors induced a similar effect at the small-RNA level. Subsequent analyses using more moderate hypoxic conditions showed large discrepancies between the change in expression of miRNA precursors and the abundance of their mature miRNAs (71). More recently, the miR319b precursor was shown to generate an additional miRNA that targets an intron-continuing form of RAP2.12 mRNAs in *Arabidopsis* leaves and inflorescences. However, the role of this miRNA in hypoxic signaling was not studied further (107).

Trans-acting small RNAs: short RNA molecules of different origins that hinder the expression of specific genes characterized by nucleotide complementarity

G-protein: guanine nucleotide-binding proteins that act as molecular switches to transduce signals from the cellular environment

CONVERGENCE AND DIVERGENCE BETWEEN LOW-OXYGEN SIGNALING PATHWAYS IN DIFFERENT EUKARYOTES

Oxygen homeostasis in animals is mediated mainly by the hypoxia-inducible factor 1 (HIF-1) transcriptional complex. This mechanism relies on a heterodimeric basic helix-loop-helix transcription factor (103). The HIF-1β subunit is constitutively expressed and stable in both hypoxic and normoxic conditions, whereas HIF-1α is directly controlled at different levels by molecular oxygen (127). In oxygenated cells, HIF-1α is rapidly degraded following hydroxylation of specific proline residues. An additional mechanism relies on asparagine hydroxylation, which hinders the association of transcriptional coactivators (62). Instead, under hypoxic conditions, HIF-1α is stabilized and transactivates specific genes involved in blood vessel development, energy metabolism, and cancer onset and progression (9).

The N-end rule pathway also plays a role in oxygen sensing in animal cells, although separate from that of the HIF machinery, and its role has been linked primarily to NO perception (47). Among the Cys2 proteins in animals, regulator of G-protein signaling 4 (RGS4) and RGS5 are controlled by the cysteine-related branch of N-end rule proteolysis (22, 64), although the initial oxidation of cysteine seems to depend only on NO and oxygen levels and not to be enzymatically catalyzed, as in plants (129). Also, in fungi, oxygen sensing relies on mechanisms not very different from those in animals and plants. In budding yeast (*Schizosaccharomyces pombe*), a membrane-tethered transcription factor (Sre1) is released into the nucleus upon hypoxia to trigger the molecular response to low oxygen (48). In contrast to plants, the dissociation of Sre1 from the plasma membrane depends on oxygen-dependent proteolytic cleavage. Additionally, a dual dioxygenase/protease, Ofd1, controls the degradation of the nuclear-targeted fragment of Sre1, although covalent modification of the target is not involved and the dioxygenase domain is directly involved in regulating the protease activity (49).

Considered together, the low-oxygen-sensing mechanisms in these three different kingdoms indicate that evolution selected a mechanism relying on direct control of the abundance and localization of transcriptional regulators (RAP2.12, HIF, and Sre1) by oxygen levels (**Figure 4**). The evolutionary convergence of oxygen sensing in animals and plants also appears in the recruitment of oxygen-dependent oxidoreductase enzymes to fine-tune the molecular response to hypoxia. Surprisingly, such a mechanism is not present in fungi, in which cysteine is not oxidized and therefore does not act as a destabilizing residue (37, 122). Compared with the HIF- and ERF-VII-dependent mechanisms, N-end rule–mediated sensing appears to be of more ancient origin, being common to both plant and animal kingdoms (37, 122).

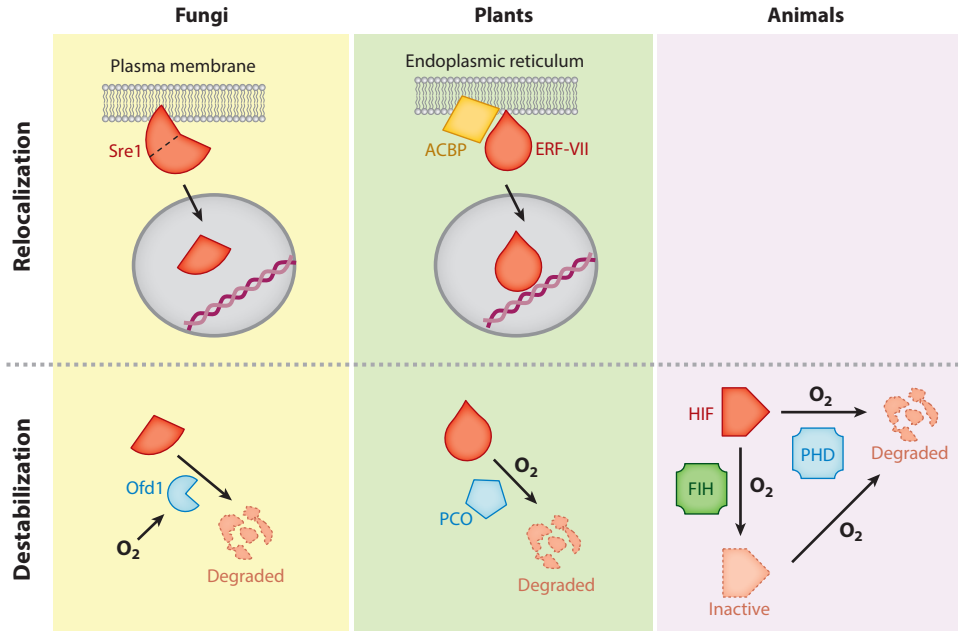


Figure 4

Cross-kingdom comparison of low-oxygen-sensing mechanisms. Oxygen-dependent relocalization of a transcription factor that acts as a master regulator of the anaerobic response (shown in *red*) from membrane systems into the nucleus is common to fungi and plants. In *Schizosaccharomyces pombe*, the transcription factor Sre1 is cleaved in an indirect oxygen-dependent manner, thereby releasing an active N-terminal fragment into the nucleus. In *Arabidopsis thaliana*, RAP2.12 is localized at the plasma membrane, where it interacts with acyl–coenzyme A (CoA)–binding proteins (ACBPs). Hypoxic conditions trigger its release by an unknown mechanism. Oxygen-dependent destabilization of the hypoxic master regulator is also shared among eukaryotes, although through different mechanisms: In yeast, Sre1 is degraded by the protease Ofd1, whose activity is directly stimulated by oxygen binding. In plants, plant cysteine oxidases (PCOs) prepare RAP2.12 for protein degradation by oxidizing the N-terminal cysteine. In animals, the HIF-1α transcription factor is hydroxylated at a proline residue in the presence of oxygen by prolyl dehydrogenases (PHDs), a modification that initiates HIF degradation by the proteasome. A second hydroxylation at an asparagine residue by FIH also inactivates the transcription factor.

SUMMARY POINTS

1. Plant tissues that are not photosynthetically active are generally hypoxic compared with their environment.
2. The hypoxic condition of a cell affects energy metabolism even before fermentation is activated.
3. A multitude of metabolic adaptations can optimize the amount of oxygen that is used to produce the ATP required for cell maintenance and growth.
4. An oxygen concentration–dependent and nitric oxide–affected proteolytic pathway determines the lifetime of proteins with a penultimate cysteine in the N-terminal amino acid sequence.

5. Group-VII ethylene response factor (ERF) transcription factors are characterized by such an N-terminal cysteine and regulate the expression of genes that are typically induced by low oxygen concentrations.
6. Proteolytic control of the lifetime of ERF-VII transcription factors by plant cysteine oxidases constitutes the core elements of the plant oxygen sensing and signaling cascade.
7. Low oxygen responses in plants are evoked not only by the oxygen concentration per se but also by the energy status and the reactive oxygen species and nitric oxide levels of a cell. The combination of all these factors is involved in regulating the (metabolic) responses to low oxygen.

FUTURE ISSUES

1. The control mechanisms of (*a*) the metabolic activity of glycolysis and the tricarboxylic acid cycle and (*b*) the mitochondrial electron transport chain require investigation. The development of stable isotope labeling for metabolic flux analysis will provide important tools for this research.
2. Detailed analysis of the specificity of the oxygen-sensing pathway and crosstalk with other pathways is needed (60).
3. Studies should investigate the physiological function of other oxygen-dependent N-end rule–mediated responses existing in plants.
4. The development of a noninvasive oxygen reporter would allow the determination of (sub)cellular oxygen concentrations with high spatial and temporal resolution.
5. Molecular events and molecules involved in the transduction of low-oxygen signals across different cellular compartments remain to be identified.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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