Chapter 10

Hypoxic Respiratory Metabolism in Plants: Reorchestration of Nitrogen and Carbon Metabolisms

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Summary

Hypoxia is a rather common phenomenon in plants that occurs naturally during development (e.g. in inner seed tissues) or due to adverse environmental conditions (waterlogging in crops). However, the specific metabolic and molecular responses to hypoxia have been disentangled only recently. Quite generally, oxygen shortage impacts on energy generation by mitochondrial metabolism. There is a conserved transcriptional response orchestrated by the so-called N end rule pathway (NERP) of proteolysis for oxygen sensing and signaling in plants. Downstream events include a deep reconfiguration of carbon metabolism that nicely illustrates the role played by biochemical enzymatic regulation as an indirect oxygen-sensing system responsible for changes in fluxes of the tricarboxylic acid (TCA) cycle, glycolysis and fermentation. Hypoxia has consequences not only for primary carbon metabolism but also for nitrogen metabolism. In fact, adaptive respiratory responses to low oxygen constraints nitrate assimilation and transaminations, and are coupled to the metabolism of nitric oxide, an endogenous signaling molecule.

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G. Tcherkez, J. Ghashghaie (eds.), *Plant Respiration: Metabolic Fluxes and Carbon Balance*, Advances in Photosynthesis and Respiration 43, https://doi.org/10.1007/978-3-319-68703-2_10,

I. Introduction

Plants, as opposed to animals, are not equipped with a tissue or a system dedicated to oxygen (O_2) uptake and delivery to the organs. As a consequence, even under aerobic conditions, dense cell packing in some tissues may lead to permanent oxygen shortage, e.g. in developing and germinating seeds, tubers, bulky fruits, meristems, germinating pollen, and phloem (van Dongen and Licausi 2015). Roots, however, are not adapted to hypoxic or anoxic environment, except in species naturally growing in waterlogged soils, swamps, etc. Current climate change is predicted to change rainfall regimes and as already observed by the GRID-Arendal center and the United Nations Environment Program (UNEP), the number of flooding events have increased all around the world in the last decades (Fig. 10.1). In this context, plants may encounter more frequently prolonged waterlogging periods (and thus oxygen shortage in the rhizosphere) due to heavy precipitations. Under such circumstances, oxygen shortage in roots comes from O_2 diffusion in water (waterlogged soils) being slower than in air and also the competition between roots and respiring microorganisms for available O_2 (Drew 1997). This situation is even worse in agricultural systems since common crop varieties have been selected to cope with various abiotic stresses such as drought and are thus not able to tolerate prolonged environmentally-driven hypoxia or anoxia (Bailey-Serres et al. 2012; Bailey-Serres and Voesenek 2008; Licausi 2013). Consequently, the adaptation to low-oxygen stress now appears to be an important aspect of crop performance. Also, it is critical to understand the responses to hypoxia at the cellular level, including metabolic acclimation and preparation to returning back to air. The knowledge of early and late molecular responses to low oxygen should provide

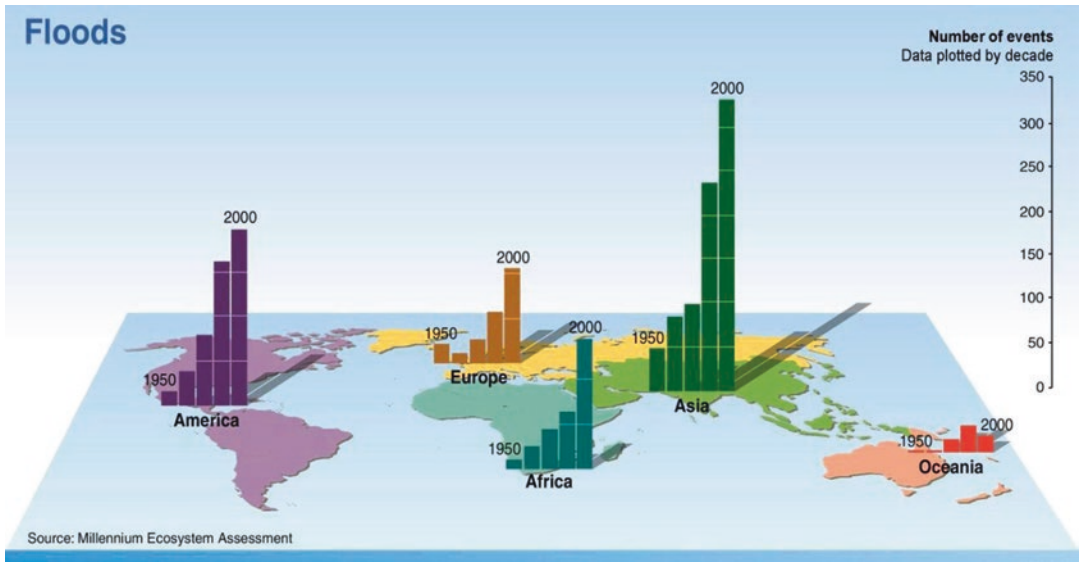

Fig. 10.1. Steady increase in the number of flood events on continents since 1950 as reported by the GRID-Arendal (http://www.grida.no/graphicslib/detail/number-of-flood-events-by-continent-and-decade-since-1950_10c2).

information on key actors and thus help geneticists to drive breeding programs for the selection of crops adapted to hypoxic rhizospheres.

Notorious damaging effects of hypoxia are associated with an energy 'crisis', that is, a reduction in respiratory ATP production and cytoplasm acidification coming from the decreased activity of the plasma membrane H^+ pumping ATPase. Transcriptome and translatome analyses in *Arabidopsis* revealed a group of 49 genes that are prioritized for translation in response to low oxygen stress (Mustroph et al. 2009). Only a few genes encoding enzymes of carbon primary metabolism and energy homeostasis were found amongst typical hypoxia-responsive genes: pyruvate decarboxylase (PDC1 and PDC2), alcohol dehydrogenase (ADH1), and sucrose synthase (encoded by SUS4) (Mustroph et al. 2009). Although nitrogen metabolism was shown to contribute to cellular acclimation to low-oxygen stress in plants (Bailey-Serres et al. 2012; Bailey-Serres and Voesenek 2008), only the gene encoding alanine aminotransferase (*AlaAT*) was found among core hypoxia-response genes (Mustroph et al. 2009). Transcriptomics analysis of rice coleoptile (using a variety adapted to elongate under anoxia) showed a response similar to that in *Arabidopsis*, with the induction of SUS4, PDC and ADH. However, genes encoding enzymes of pyruvate and phospho*enol*pyruvate metabolism were found to be the most affected (Lasanthi-Kudahettige et al. 2007): while the expression of genes encoding pyruvate kinase (PK) and pyruvate dehydrogenase (PDH) were almost not changed, genes encoding pyruvate phosphate dikinase (PPDK) and phospho*enol*pyruvate carboxykinase (PEPCK) were highly expressed and the transcription of the gene encoding phospho*enol*pyruvate carboxylase (PEPC) was strongly inhibited (Lasanthi-Kudahettige et al. 2007). It is worth noting that in rice coleoptile under aerobic condition, PEPCK was almost not expressed while PEPC transcripts were abundant (Lasanthi-Kudahettige et al. 2007).

In animals, the adaptive response is orchestrated very early at the onset of hypoxic stress by a direct oxygen sensing mechanism based on oxygen-dependent posttranslational hydroxylation of a hypoxia-inducible factor (HIF subunit α) (Kaelin and Ratcliffe 2008). When oxygen is available, HIFα is hydroxylated, allowing polyubiquitynation and degradation by the proteasome. At low oxygen mole fraction, HIFα is not hydroxylated and escapes degradation, homodimerizes and the dimer translocates to the nucleus where it transcriptionally activates up to 200 genes, including genes involved in erythropoiesis, angiogenesis, autophagy, and energy metabolism (Kaelin and Ratcliffe 2008). In plants, neither such an early and massive reaction nor a similar mechanism of direct oxygen sensing has been found (Bailey-Serres et al. 2012). In the last decade, the breakthrough in the area of plant response to low-oxygen stress was the discovery of the role played by a family of transcription factors belonging to group-VII Ethylene Response Factors (ERFs): RAP2.2, RAP2.3, RAP2.12, HRE1, and HRE2 (for a review, see Bailey-Serres et al. 2012; Limami et al. 2014; van Dongen and Licausi 2015). It has been proposed that at least two members of the group-VII ERFs, RAP2.2 and RAP2.12, are constitutively expressed and act redundantly as principal activators of genes encoding proteins involved in the response to low-oxygen stress (Gasch et al. 2016). However, all of the members of group-VII ERFs are subjected to oxygen-dependent posttranslational modification

through the N-end rule pathway (NERP) for protein proteolysis (Gibbs et al. 2011; Licausi et al. 2010, 2011). After N terminal methionine cleavage by a methionine aminopeptidase, the exposed cysteine is oxidized to Cys-sulfonic acid by O_2. An arginine residue is added to the oxidized cysteine by an arginyl tRNA transferase and the argininylated protein is then recognized by an N-recognin [PROTEOLYSIS 6 (PRT6) in *Arabisopdis thaliana*], which polyubiquitinates the protein thus allowing its degradation by 26S proteasome (Licausi et al. 2013). When cellular oxygen concentration decreases below a (yet unknown) threshold, RAP2.2 and RAP2.12 would escape post-translational modification and degradation. They are then transported to the nucleus where they induce the expression of secondary ERFs such as *HRE1* and *HRE2,* allowing several downstream hypoxia-response genes to be expressed (Licausi 2013; Sasidharan and Mustroph 2011). Upon the return to normoxia, all of the members of group VII ERFs are subjected to posttranscriptional-mediated proteolysis. Members of the ERF transcription factors family involved in the regulation of hypoxia responsive genes have been also identified in rice (SUB1A, SK1 and SK2) (Hattori et al. 2009; Xu et al. 2006). Characterization of *Arabidopsis* mutants affected in the NERP pathway of proteolysis showed that *PDC* and *ADH* genes, but not *AlaAT*, are under the control of group-VII ERFs (Gibbs et al. 2011; Licausi et al. 2011). We thus hypothesize that the acclimation response to hypoxia is established gradually during the oxygen shortage period. This would reflect the fact that plants can survive long periods of oxygen shortage (up to several hours or days of waterlogging) as compared to animals (Drew 1997). That is, before cellular events involving group VII-ERFs are triggered, the prime response involves indirect sensing of low oxygen via *(i)* the impairment of the mitochondrial electron transfer chain and changes in redox status (NADH/NAD ratio), *(ii)* reactive oxygen species (ROS), *(iii)* nitric oxide (NO), and *(iv)* energy status of the cell (ATP content). Afterwards, transcriptionally-controlled responses that depend on NERP proteolysis turn-over of ERF-VII transcription factors are activated to amplify the shift from aerobic to anaerobic metabolism and trigger long-term effects such as hormone-dependent stem elongation and aerenchyma development (Bailey-Serres et al. 2012; Bailey-Serres and Voesenek 2008; Drew 1997; Limami et al. 2014; van Dongen and Licausi 2015).

Taken as a whole, hypoxia is expected to cause major changes in both metabolome and transcriptome, associated with changes in metabolic fluxes in the tricarboxylic acid (TCA) cycle, glycolysis and fermentation. In this chapter, we will first describe these effects and provide an overview of recent findings dealing with the reorchestration of carbon and nitrogen metabolisms under hypoxia. Second, considering the effects of hypoxia for nitrate assimilation, we will discuss the cellular control of hypoxic nitrogen metabolism and the involvement of nitric oxide, an endogenous signaling molecule derived therefrom.

II. Reconfiguration of C and N Metabolisms Under Hypoxia

A. Inhibition of Photosynthesis

Waterlogging-induced hypoxia has been described in the literature as being a possible cause of photosynthesis impairment (Kozlowski and Pallardy 1984). One of the reported reasons is that the drop in photosynthesis is associated with stomatal closure (Huang et al. 1994; Malik et al. 2001; Mielke et al. 2003; Mollard et al. 2010; Striker et al. 2005; Vu and Yelenosky 1991). Stomatal closure may occur under hypoxic conditions in response to leaf dehydration induced by water transport impairment which is in turn

caused by oxygen deficiency in roots (Else et al. 2001). However, stomatal closure can also occur without noticeable changes in leaf water potential but rather, as a response to hormonal regulation regardless of plant water status. In fact, available evidence supports the idea of stomatal closure mediated by abscisic acid (ABA) in flooded plants (Else et al. 1996; Jackson and Hall 1987). In crops, prolonged hypoxia leads to a decrease in net photosynthesis rate of mesophyll cells *per se* (Yordanova and Popova 2001) and thus in biomass production, leaf size and yield (Kozlowski and Pallardy 1984; Schlüter and Crawford 2001). This long-term effect of hypoxia on photosynthetic capacity has been explained by the degradation of chlorophylls and other components of the photosynthetic apparatus, following early leaf senescence in response to nitrogen deficiency that occurs in leaves of waterlogged plants (Grassini et al. 2007; Manzur et al. 2009; Yordanova and Popova 2001). Also, waterlogged plants exhibit carbohydrate accumulation in leaves, likely due to the reduction in sugar utilization resulting from altered growth and phloem transport (Barta 1987; Wample and Davis 1983). Sugar accumulation exerts in turn a negative feedback on photosynthesis rate (Pego et al. 2000).

B. Cellular Energy Generation and Sugar Provision

Changes in both nitrogen and carbon primary metabolism as a consequence of hypoxia have been reported in many species belonging to plants, animals (including *Homo sapiens*), fungi and bacteria (Mustroph et al. 2010). Conserved changes are described as adaptive reactions at the cellular level allowing both the mitigation of detrimental effects of low-oxygen stress and the preparation to returning to air. At the cellular level, damaging effects are related to an energy 'crisis', that is, the reduction in respiratory ATP production and cytoplasm acidification as a consequence of impaired plasma membrane H^+ pumping ATPase. In plants, faster glycolytic activity coupled to alcoholic fermentation (and the associated loss of carbon to the medium as ethanol) is responsible for carbon reserves exhaustion. As a consequence, it is widely accepted that hypoxic tissues experience a carbon-starvation stress in addition to low-oxygen stress. Therefore, carbohydrate supply to hypoxic roots during prolonged flooding periods appears to be crucial for plant survival (Drew 1997; Jaeger et al. 2009). Accordingly, amylases have been shown to be active under hypoxic or anoxic conditions in hypoxia-tolerant rice seeds, and inactive (inhibited) in hypoxia-sensitive seeds of wheat and barley (Guglielminetti et al. 1995).

C. Metabolic Pathways Under Hypoxia

A general model of the metabolic response to hypoxia can be drawn from published data that used metabolomics sometimes coupled to ^{13}C and ^{15}N isotope labeling, in various species such as *Arabidopsis thaliana,* soybean (*Glycine max*), bird's foot trefoil (*Lotus japonicus*), barrel clover (*Medicago truncatula*) and rice (*Oryza sativa*). As stated above, starch and sucrose degradation are activated under hypoxia to meet the increased carbon demand by glycolysis. As a strategy of ATP saving and increasing the energetic balance of glycolysis, pyrophosphate (PPi)-dependent kinases are preferred over ATP-dependent kinases in hypoxic tissues. In contrast with ATP, PPi does not come from the mitochondrial electron chain but is a by-product of many reactions, like DNA, RNA, proteins and cellulose biosynthesis or PEP synthesis by pyruvate Pi dikinase. It is also produced during β-oxidation of fatty acids by thiokinase (Ferjani et al. 2012). Sucrose degradation and hexose phosphorylation occur preferentially via sucrose synthase and UDP-glucose pyrophosphorylase (UGPase). Sucrose synthase cleaves sucrose in the presence of UDP, thereby generating UDP-

glucose and fructose. Fructose can then be phosphorylated by hexokinase, using one ATP. UDP-glucose could be used along with PPi by UGPase to generate glucose-1-phosphate and UTP. This pathway thus potentially generates one phosphorylating equivalent (UTP) and also consumes one (ATP). Simple sucrose cleavage by invertase would cost two ATP, required to phosphorylate both glucose and fructose. Also under hypoxia, fructose-1,6-bisphosphate synthesis is catalyzed by PPi-fructose 6-phosphate 1-phosphotransferase (PPi-dependent phosphofructokinase, PFP) instead of ATP-dependent phosphofructokinase (PFK). Also, pyruvate synthesis might be supplemented by PPDK (although the PPDK-catalyzed conversion of PEP to pyruvate is unfavorable under physiological conditions) in addition to PK, but this hypothesis needs to be tested. Enzymes catalyzing other steps of glycolysis are also stimulated under low oxygen. So is the case of fructose-1,6-bisphosphate aldolase, triose phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase.

The fate of pyruvate under hypoxic conditions is primarily fermentation to ethanol, which regenerates NAD^+ to maintain substrate-level ATP production (i.e., glycolysis). Lactate dehydrogenase (LDH) is also known to be activated in hypoxic tissues. However, it seems that PDC activity prevails over LDH so that fermentative NAD^+ regeneration via PDC + ADH rather than LDH appears to be specific to plants (Bailey-Serres and Colmer 2014). Pyruvate is also converted to alanine, sometimes to considerable levels (Fig. 10.2). As such, alanine accumulation is described as a hallmark of hypoxic tissues in many plant species. Also in *Arabidopsis*, alanine aminotransferase (*AlaAT*) is the sole gene associated with nitrogen metabolism found in hypoxia-responsive genes (Mustroph et al. 2009). This might appear rather surprising since alanine production does not regenerate NAD^+, and alanine plays neither a signaling nor protective role. What is the rationale of alanine accumulation then? Tentative explanations have been provided by several authors (for a review, see Bailey-Serres and Voesenek 2008; Limami et al. 2014) and completed recently by Diab and Limami (2016). They suggested that alanine must be viewed as a masterpiece in the jigsaw puzzle of hypoxic carbon and nitrogen metabolisms: alanine and glutamate are involved in a AlaAT/NADH-GOGAT cycle whereby NAD^+ is regenerated and the storage of glycolytic products in the form of alanine (rather than being lost in the form of ethanol) saves organic carbon. In such a cycle, the reaction sequence is simply the following: pyruvate + glutamate → alanine + 2-oxoglutarate and 2-oxoglutarate + glutamine + NADH → 2 glutamate + NAD^+. That way, NADH is re-oxidized using *indirectly* pyruvate generated by glycolysis (Fig. 10.2). Evidence has been provided for NADH-GOGAT activity under hypoxia. For example, NADH-GOGAT activity has been shown to increase in hypoxic *Medicago truncatula* roots (Limami et al. 2008). In addition, when roots of *Medicago truncatula* or soybean were fed with $^{15}NH_4^+$ with or without methionine sulfoximine (MSX, a potent glutamine synthetase inhibitor), ^{15}N-glutamate was synthesized only in the absence of MSX (Antonio et al. 2016; Limami et al. 2008). The involvement of the AlaAT/NADH-GOGAT pathway holds if there is a source of glutamine to sustain GOGAT activity. Should glutamine be synthesized de novo by glutamine synthetase, this pathway would consume extra ATP and this is probably not desirable under hypoxic conditions. It thus appears more likely that glutamine originates from proteolysis, which is effectively enhanced under hypoxia. Future studies are warranted to provide more information on this, for example using a precise ^{15}N mass balance.

Diab and Limami (2016) also proposed that alanine may represent a readily utilizable carbon and nitrogen store upon reoxygenation (normoxia) before carbon fixation via photosynthesis is fully reestablished. That is, alanine would be remobilized

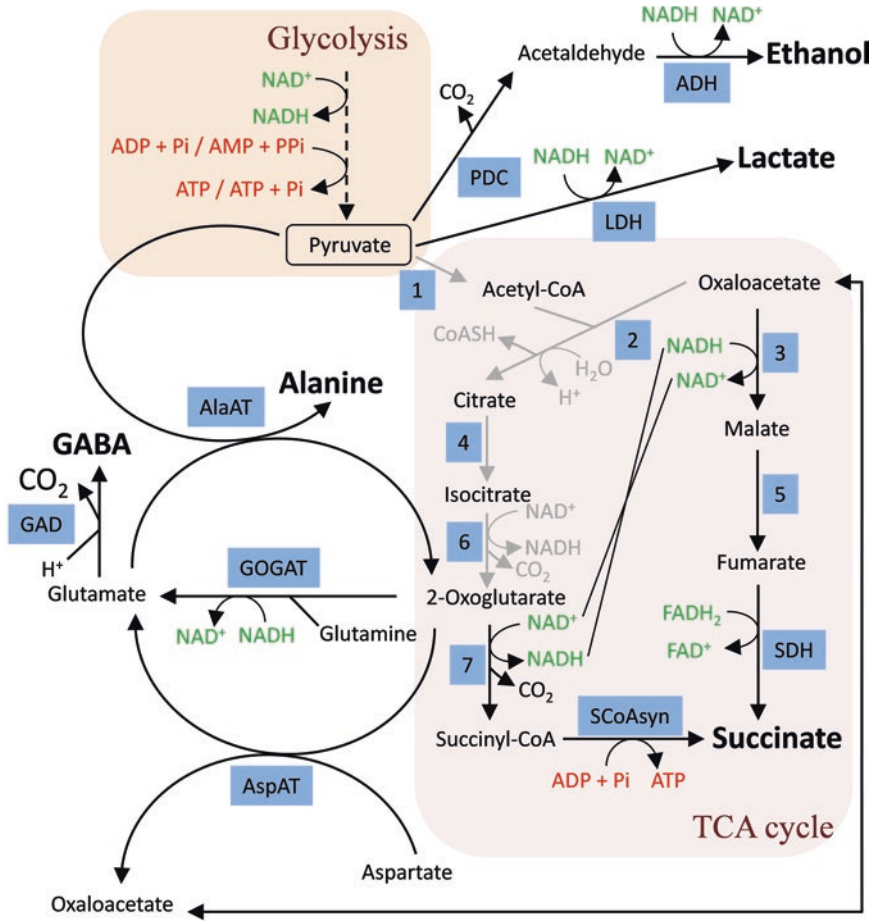

Fig. 10.2. **Overview of primary metabolism in tissues subjected to oxygen deficiency.** Under low O_2 conditions, cell metabolism has to counteract a reduction in respiratory ATP production and cytoplasm acidification. Glycolysis becomes the main source of ATP synthesis. To keep glycolysis going, NAD^+ must be continuously regenerated and pyruvate (encircled) accumulation should be prevented. This is mainly achieved by channeling pyruvate to lactate *via* lactate dehydrogenase (LDH) and to ethanol *via* pyruvtae decarboxylase (PDC) and alcohol dehydrogenase (ADH). Also to a lower extent, pyruvate is committed to an alanine aminotransferase (AlaAT) coupled to glutamine 2-oxoglutarate aminotransferase (GOGAT) that regenerates NAD^+ or aspartate aminotransferase (AspAT) that produces oxaloacetate. 2-oxoglutarate and oxaloacetate could enter in the TCA cycle reorganized in two independent branches leading to succinate accumulation. This allows the regeneration of FAD^+ *via* succinate dehydrogenase (SDH) and ATP synthesis *via* succinyl-CoA synthetase (SCoAsyn). Cytoplasm acidosis could be partly compensated for by the consumption of protons by glutamate decarboxylase (GAD). Dashed, continuous and gray arrows stand for multistep pathways, reactions stimulated under oxygen deficiency, and reactions inhibited during oxygen deficiency, respectively. Metabolites shown in bold are accumulated.*1*, pyruvate dehydrogenase; *2*, citrate synthase; *3*, malate dehydrogenase; *4*, aconitase; *5*, fumarase; *6*, isocitrate dehydrogenase; *7*, 2-oxoglutarate dehydrogenase.

through an AlaAT/GDH recycling whereby alanine is reconverted to pyruvate and glutamate + NAD^+ oxidized to 2-oxoglutarate + NADH + NH_4^+ by glutamate dehydrogenase (GDH). Then NADH and pyruvate would be channeled to the mitochondrial electron chain and the TCA cycle, respectively.

γ-aminobutyric acid (GABA) has also been shown to be a metabolite that typically accumulates under hypoxia (Fig. 10.2).

However, GABA is not a static end product since it is produced by glutamate decarboxylase (GAD) and consumed by GABA aminotransferase (GABA-T). On the one hand, these reactions are part of the GABA shunt which allows glutamate to be recycled by the TCA cycle. Furthermore, GDC activity contributes to acidosis mitigation because GABA synthesis consumes protons (glutamate + $H^+ \rightarrow CO_2$ + GABA). On the other hand, glutamate recycling via the GABA shunt has long been considered to be unlikely because succinic semialdehyde (the product of GABA-T) conversion to succinate (catalyzed by succinate semialdehyde dehydrogenase, SSDH) requires NAD^+. Furthermore, the optimal pH for SSDH catalysis is not compatible with the observed drop in cytosolic pH during hypoxia (Rocha et al. 2010). Nevertheless, recent experiments of ^{15}N redistribution have suggested the involvement of the GABA shunt (Antonio et al. 2016).

D. Carbon Fluxes Under Hypoxia

In the last decade, some advance has been provided by isotopic labeling. ^{13}C analyses in metabolites after ^{13}C-glutamate and ^{13}C-pyruvate labeling in soybean waterlogged roots indicated that succinate is one, if not the major product of glutamate metabolism via the AlaAT/NADH-GOGAT cycle. This effect simply comes from 2-oxoglutarate, the product of glutamate deamination, being oxidized by 2-oxoglutarate dehydrogenase (2OGDH) and then succinyl-CoA synthase, thereby producing ATP (Antonio et al. 2016). The close coupling between alanine production and the TCA cycle is corroborated by the finding that the gene encoding the mitochondrial isoform of AlaAT is up-regulated under hypoxia in *Medicago truncatula* (Ricoult et al. 2005).

In bacteria, lower eukaryotes, shellfish, and cancer cells (reviewed by Chinopoulos 2013) succinate build-up is also explained by the following sequence: 2-oxoglutarate + aspartate → glutamate + oxaloacetate and oxaloacetate → malate → fumarate → succinate (Fig. 10.2). In plants, only partial information is available on this hypothetical segment of the TCA cycle and further work is needed to elucidate this pathway. For example, ^{13}C-labeling experiments in soybean and *Lotus japonicus* showed that while malate and succinate were significantly labeled (Antonio et al. 2016; Rocha et al. 2010) no ^{13}C enrichment was detected in fumarate. The authors strongly defended the idea that malate might derive from oxaloacetate (OAA) by the action of malate dehydrogenase (MDH) rather than from fumarate because of the inhibition of succinate dehydrogenase (SDH, complex II of the mitochondrial electron transport chain) due to the saturation of the ubiquinone pool in the absence of oxygen (Rocha et al. 2010).

Uncertainty also remains about the metabolic origin of OAA. Rocha et al. (2010) proposed that in waterlogged roots of *Lotus japonicus*, aspartate aminotransferase (AspAT) produces OAA but this proposal was not supported by isotopic labeling. Recently, the same research group fed soybean roots under hypoxia with ^{13}C- and ^{15}N-labeled molecules and obtained surprising results. Both ^{13}C-glutamate and ^{13}C-pyruvate labeling experiments led to the appearance of ^{13}C-aspartate and ^{13}C-malate. At first glance, this suggests that malate was mostly synthesized by the TCA cycle. It contradicts the assumption that succinate dehydrogenase is inhibited and MDH operates in the reductive direction (malate synthesis) under hypoxia. The simplest explanation of this contradiction is that $^{13}CO_2$ derived from ^{13}C-pyruvate and ^{13}C-2-oxoglutarate decarboxylation has been re-assimilated by the PEPC thereby producing ^{13}C-OAA. OAA has then been aminated to ^{13}C-aspartate by AspAT or reduced to malate by MDH. Actually, malate seems to be a more likely end product than aspartate under hypoxic

conditions, because $^{15}NH_4^+$ labeling leads to very low ^{15}N redistribution in aspartate (and asparagine) showing that aspartate synthesis by AspAT has a very low activity (Antonio et al. 2016).

Further experimental work is needed to confirm (or invalidate) this plausible, PEPC-based explanation. In particular, the gene encoding PEPC has never been listed amongst visibly up-regulated genes transcriptomics studies under hypoxia, and it has been shown to be strongly down-regulated in hypoxic rice coleoptiles (Lasanthi-Kudahettige et al. 2007). Having said that, the PEPC hypothesis cannot be ruled out considering many examples of enzymes for which gene expression (mRNA abundance) decreased while the activity remained constant or even increased under hypoxia as compared to normoxia. For example, in several studies conducted on rice coleoptiles and seedlings, the expression of genes encoding MDH and 2OGDH have been shown to decrease although enzyme activities were present, with 2-oxoglutarate effectively converted to succinyl-CoA further metabolized to succinate, thus producing ATP and regenerating NAD^+.

III. Involvement of Nitric Oxide in Low-Oxygen Stress Tolerance

A. Nitric Oxide Production Under Oxygen Deficiency

At both molecular and physiological levels, low oxygen in roots may affect nutrient assimilation including nitrogen (Narsai et al. 2011). One of the metabolic responses to oxygen deficiency is nitric oxide (NO) accumulation. In the past few years, this gaseous free radical has emerged as an important signaling molecule involved in several plant physiological and developmental processes, and adaptive responses to biotic and abiotic stresses (Besson-Bard et al. 2008). Due to its free radical nature (single unpaired electron), NO has a short half-life (in the order of seconds) and can react easily with oxygen/superoxide or with thiol- and transition metal-containing proteins. An increase in NO emission has been reported to occur under low oxygen in various species, such as *Arabidopsis thaliana* (Hebelstrup et al. 2012), *Chlorella sorokiniana* (Tischner et al. 2004), *Medicago sativa* (Dordas et al. 2003), *Medicago truncatula* (Horchani et al. 2011), *Nicotiana tabacum* (Planchet et al. 2005), *Nicotiana sylvestris* (Shah et al. 2013), *Populus sp.* (Liu et al. 2015) or *Zea mays* (Mugnai et al. 2012). In plants, there are two main NO biosynthetic pathways (Gupta et al. 2011): reductive (nitrite-dependent reaction) and oxidative (L-arginine-dependent reaction). Since L-arginine-dependent NO production via both NO synthase-like enzymes or hydroxylamine- and polyamine-degradation requires oxygen, the nitrite-dependent pathway is believed to be the most active under low-oxygen stress.

In nitrite-dependent NO production in hypoxic roots, nitrate reductase (NR) is the first and the best-characterized step. This cytosolic enzyme catalyzes not only the reduction of nitrate to nitrite (leading to a proton consumption and $NAD(P)^+$ regeneration), but also the reduction of nitrite to NO (Rockel et al. 2002). By following NO emission from purified NR or from intact leaves under nitrogen (atmosphere made of 100% N_2), the rate of NO production has been found to represent less than 1% of NR activity (Planchet et al. 2005). It should be noted that the K_m for nitrite is estimated to be near 100 μM and the reaction is competitively inhibited by nitrate (K_i=50 μM), suggesting that nitrite and nitrate are reduced by NR at the same active site (Kaiser et al. 2002; Rockel et al. 2002). Compared to ammonium-cultivated or tungstate-treated plants in which NR activity is suppressed, nitrate-fed plants produced higher amounts of both nitrite and NO during hypoxia or anoxia (Lea et al. 2004; Liu et al. 2015; Oliveira et al. 2013a; Planchet et al. 2005; Tischner

et al. 2004). The involvement of NR has been further confirmed using NR-deficient mutants which don't accumulate nitrite and are unable to produce NO under anaerobic conditions (Planchet et al. 2005). The increase in NO synthesis under low oxygen, which is 100–1000 fold higher than under aerobic conditions, correlates to nitrite accumulation which results from both *(i)* the stimulation of NR activity by post-translational modification (dephosphorylation on a conserved serine residue) triggered by cytoplasmic acidosis (Botrel and Kaiser 1997), and *(ii)* the concomitant inhibition of nitrite reductase (NiR) (Botrel et al. 1996). The high NR-dependent NO production under hypoxia could explain the beneficial effect of nitrate through the regeneration of $NAD(P)^+$ which is required for glycolysis. In fact, nitrate reduction by NR has been suggested to alleviate some of the effects of oxygen deficiency in diverse plant species during hypoxia (Allegre et al. 2004; Botrel et al. 1996; Horchani et al. 2011; Oliveira et al. 2013b).

Another enzyme, the plasma membrane-bound nitrite:NO reductase (NI-NOR), is able to form NO from nitrite reduction using cytochrome c (instead of NADH) as an electron donor under very low oxygen mole fraction (Stohr et al. 2001). In the apoplast, nitrite comes from secretion from the cytosol via transporters as well as nitrate reduction by plasma membrane-bound NR (PM-NR) which is differentially regulated as compared to cytosolic NR. The root-specific form of PM-NR uses succinate as an electron donor and needs acidic conditions (optimum activity at pH 6.1). Nitrite-dependent NO production has been *(i)* shown to occur in root apoplast of *N. tabacum* after activation of apoplastic NR under oxygen deficiency and *(ii)* suggested to be regulated by the availability of oxygen *in vivo*, because NI-NOR activity is reversibly inhibited by oxygen (loss of 78% of the activity in ambient air) (Stohr and Stremlau 2006). Thus, it has been proposed that apoplastic NO production plays the role of a signal associated with oxygen deficiency (Stohr and Stremlau 2006).

Although NR has been described to be the main NO source in higher plants, mitochondria have also been shown to produce NO from nitrite using NADH as a reductant when dissolved oxygen is low or absent (Planchet et al. 2005; Tischner et al. 2004). However, the mechanism of nitrite transport into mitochondria is still not well understood. Using inhibitors of the mitochondrial electron transport chain, nitrite-dependent NO generation in hypoxic or anoxic plant cells has been shown to occur at specific sites, such as complex IV (cytochrome c oxidase; COX), complex III (cytochrome bc1, cytochrome c reductase) and alternative oxidase (AOX) (Planchet et al. 2005; Stoimenova et al. 2007). Nevertheless, NO-generating mitochondrial activity in the absence of oxygen represents less than 1% of respiratory electron transport capacity of purified root mitochondria, and the K_m value for nitrite is estimated to be near 175 μM (Gupta et al. 2005). It should be noted that the low rate of mitochondrial NO formation could also be explained by the high NO reactivity (to be scavenged or oxidized) leading to an underestimation of NO formation. Nitrite-dependent NO production has been shown to occur at a higher rate in root mitochondria than in leaf mitochondria (Gupta et al. 2005), suggesting a specific role of NO in oxygen sensing and signaling in waterlogged plant roots.

B. Nitric Oxide, an Important Regulator of Plant Respiration Under Low-Oxygen Stress

Mitochondria of plants subjected to strict anoxic conditions show modifications in enzyme composition, but not in their ultrastructure or metabolic activity unless nitrate is withdrawn from the medium (Vartapetian et al. 2003). This suggests that nitrate is a suppressor of the mitochondrial hypoxic

response, via a role in signaling or as a substrate. In principle, nitrate could be a mitochondrial terminal electron acceptor, but no evidence has been provided so far. Nitrate has also been suggested to act indirectly via nitrite, which in turn would be an electron acceptor sustaining NAD(P)H re-oxidation (Igamberdiev and Hill 2004) and anaerobic mitochondrial ATP synthesis (Stoimenova et al. 2007). In fact, the involvement of nitrite has been shown in nitrate-mediated modulation of fermentative metabolism in soybean roots under oxygen deficiency (Oliveira et al. 2013b). In this study, ethanol and lactate content decreased while NO production was stimulated upon exogenous nitrite supply to root segments detached from plants cultivated with ammonium (not nitrate) as a nitrogen source. This shows a negative correlation between NO production and the metabolic flux in fermentation. Furthermore, nitrite-dependent NO emission was sensitive to potassium cyanide (a potent inhibitor of complex IV). Although mitochondrial nitrite-dependent NO production occurs under hypoxia or anoxia, long-term *in vivo* exposure of mitochondria to NO leads to typical mitochondrial dysfunctions. NO can react with various proteins of the respiratory chain containing thiol groups and transition metals (Fe in iron-sulfur centers and haem) causing post-translational modifications such as S-nitrosylation and metal-nitrosylation and thus an impairment of respiration. Accordingly, hypoxia has been shown to induce an increase in S-nitrosylated compounds in *A. thaliana* roots (Hebelstrup et al. 2012).

1. Nitric Oxide Mitochondrial Targets

NO can have a dual effect on cell energetics depending on its mitochondrial targets. Here, two main targets are discussed: the mitochondrial electron chain and aconitase (enzyme of the TCA cycle).

NO is a reversible inhibitor of cytochrome oxidase (COX, complex IV) by competing with oxygen at the binuclear center, and thus interfere with respiratory chain activity (Cooper 2002). At low oxygen concentration, NO is an alternative electron acceptor and interacts with either the ferrous haem iron atom or oxidized cupper of COX (but not both simultaneously). The K_m for O_2 of COX has been reported to be within the order of 140 nM (Millar et al. 1994) and could rise to 1 μM after NO inhibition (Cooper 2002). It is worth noting that the inhibition coefficient of O_2 (K_i) for mitochondrial NO production is about 150 nM, which is very close to the K_m for oxygen of COX (Gupta et al. 2011). Since NO accumulates under hypoxia, the inhibitory effect of NO on COX has been shown to lead to a decrease in *(i)* oxidative phosphorylation (Yamasaki et al. 2001) and thus *(ii)* metabolic response to low oxygen (Geigenberger et al. 2000). NO-induced COX inhibition may effectively contribute to decrease oxygen consumption since NO has been demonstrated to induce an increase in intratissular O_2 in seeds (Borisjuk et al. 2007). Therefore, the control of respiratory activity and oxygen concentration by endogenous NO is likely mediated by its binding to COX.

That said, the mitochondrial alternative oxidase (AOX) is insensitive to NO. On the one hand, the K_m of AOX for O_2 is about 10 μM thus AOX activity is probably modest at low oxygen (Affourtit et al. 2001). But on the other hand, AOX is essential when the cytochrome pathway is slowed down by limiting O_2 concentration. In general, it is believed that AOX, while not contributing to proton gradient formation, reduces electron pressure on the ubiquinone pool thus preventing superoxide generation (Cvetkovska and Vanlerberghe 2012). This means that nitrite-dependent NO production strictly depends on AOX activity, which can regulate the commitment of electrons to complexes III and IV and thus to nitrite. The maintenance of mitochondrial electron flow through AOX, even at high NO concentrations, is considered to be a key aspect of

plant survival under oxygen deficiency (Gray et al. 2004; Millar and Day 1996). Transcription of genes encoding AOX has been shown to be up-regulated by NO and by the increase in citrate, which comes from NO-mediated inhibition of aconitase under hypoxic conditions (Gray et al. 2004; Gupta et al. 2012). It has been suggested that the induction of genes encoding AOX under hypoxia anticipates the increased production of reactive oxygen species (ROS) naturally occurring during reoxygenation following hypoxia (Maxwell et al. 1999).

Aconitase (aconitase hydratase) catalyzes the reversible isomerization of citrate to isocitrate via a cis-aconitate intermediate. The cytosolic isoform of aconitase is affected by NO. In fact, Gupta et al. (2012) have shown that under hypoxic conditions, the citrate content in WT plants increased by 70% compared to aerobic conditions, whereas in NR-deficient plants, it was strongly reduced (almost twofold). The citrate content was negatively correlated to aconitase activity. Interestingly, NO-induced inhibition of aconitase is more effective at low pH (Gardner et al. 1997), a condition that occurs under hypoxia. From a chemical point of view, NO interferes with the iron-sulfur (4Fe-4S) center of the protein leading to metal (Fe) nitrosylation. The inhibition of aconitase activity by NO leads to *(i)* a decrease in TCA cycle activity and thus cellular energy metabolism, *(ii)* a decrease in ROS generation due to a reduced electron flow through the mitochondrial electron transport chain (Navarre et al. 2000), *(iii)* an increased supply in organic acids, due to the outflow of citrate from the TCA cycle, and *(iv)* a decrease in 2-oxoglutarate availability, which is the substrate for nitrogen assimilation into amino acid (Gupta et al. 2012).

2. *Involvement of Nitric Oxide in Maintaining Energy and Redox Status*

Obviously, hypoxia affects mitochondrial ATP generation due to the lack of O_2 as a terminal electron acceptor. ATP generation by glycolysis and fermentation is not very efficient since the ATP yield (ATP produced per molecule of glucose) is much less than that generated aerobically by the mitochondrial electron transport chain. As stated above, nitrite-dependent NO production inhibits O_2 consumption by complex IV and thus indirectly down-regulates respiratory O_2 consumption. This in turn may avoid rapid oxygen depletion, especially in seeds and seedlings development, thus postponing complete anoxia. In other words, NO could be beneficial under hypoxic conditions to maintain minimal O_2 and ATP production by slowing down the respiration (Borisjuk et al. 2007). Under such an assumption, NO concentration would have to be controlled rather than simply accumulated. As a matter of fact, NO concentration is believed to be controlled by haemoglobins (Hb), although plant Hbs are absent from mitochondria and are located in the cytosol and the nucleus (Dordas et al. 2003; Igamberdiev and Hill 2004).

Class-1 non-symbiotic haemoglobin (nsHbs), also called phytoglobins in plants, can bind O_2 at very low concentration. The O_2 dissociation constant is estimated to be within the range 2–3 nM. That is, they can bind O_2 at concentrations two orders of magnitude below those required for saturating COX (Igamberdiev et al. 2014). Therefore, at concentrations where oxyhaemoglobin (nsHb-O_2) dissociates (below 2 or 3 nM O_2), COX does not consume oxygen. nsHbs can also scavenge NO due to the high propensity of NO to react with the redox-active cysteine residue within the distal haem pocket. This suggests that NO degradation by nsHbs may occur at oxygen concentrations that are low enough to impair mitochondrial respiration. The induction of nsHb gene expression has been shown to occur under hypoxia (Liu et al. 2015; van Dongen et al. 2009), but also after nitrate, nitrite and NO treatments and disruption of ATP synthesis (Nie and Hill 1997; Ohwaki et al. 2005). Interestingly,

HB1 overexpressed in *Arabidopsis* contributed to increase plant tolerance and survival under hypoxia, with a reduction in NO concentration and constant ATP level. Conversely, nsHb-underexpressing lines accumulated NO to a concentration 2.5-fold higher than in nsHb-overexpressing lines, and were less tolerant to hypoxia (Dordas et al. 2003). These results show that nsHbs prevent excessive hypoxia-induced mitochondrial NO production and thus presumably, also nitrosative stress (Perazzolli et al. 2004).

The mechanism of NO detoxification probably starts with diffusion across the mitochondrial membrane into the cytosol. NO is then converted (oxidized) to nitrate by oxyhaemoglobin (denoted as nsHb[Fe^{2+}]O_2) (Fig. 10.3). nsHb[Fe^{2+}]O_2 is in turn converted to methaemoglobin (in which Fe^{2+} is oxidized to Fe^{3+}; it is denoted as nsHb[Fe^{3+}]). The redox half-equations considered are as follows: nsHb[Fe^{2+}]O_2 + 4 H^+ + 3 e^- → nsHb[Fe^{3+}] + 2 H_2O ($E^0 \approx 1.10$ V) and NO + 2 H_2O → NO_3^- + 4 H^+ + 3 e^- ($E^0 = 0.96$ V). nsHb[Fe^{2+}] is regenerated from nsHb[Fe^{3+}] via a cytosolic monodehydroascorbate-dependent reductase (methaemoglobin reductase) that yields $NAD(P)^+$ (Igamberdiev et al. 2006). In the cytosol, nitrate generated from NO oxidation by haemoglobin may be reduced by NR to nitrite, also leading to $NAD(P)^+$ formation. Nitrite is then believed to be transferred back to the mitochondrial matrix through an unknown transporter (Horchani et al. 2011) and could serve as a substrate for mitochondrial NO production or terminal electron acceptor with NAD(P)H as electron donors (Stoimenova et al. 2007). These reactions thus define a cycle where NO is produced, bound to nsHbs, degraded and then eventually reformed.

At this stage, the yield of ATP production is important to consider so as to appreciate the involvement of NO in respiratory metabolism. The yield of anaerobic ATP production per NAD(P)H oxidized is about one in hypoxia-resistant plants (like rice) and is lower (around 0.7) in hypoxia-sensitive plants (barley) (Stoimenova et al. 2007). This means that for a given redox ratio NAD(P)H/$NAD(P)^+$, the ATP/ADP ratio is maintained in rice under hypoxia while in barley, NADH re-oxidation event is less efficiently coupled to ATP generation due to, e.g. AOX or alternative, non-proton pumping electron transfers. Mitochondrial oxidation of cytosolic NADH and NADPH and electron transfer to ubiquinone can also be catalyzed by non-proton pumping dehydrogenases present on the external side of the inner mitochondrial membrane (so-called "external" NAD(P)H dehydrogenases). External NAD(P)H dehydrogenases are stimulated at high calcium (Ca^{2+}) concentration (Fig. 10.3), a cellular condition observed under hypoxia (Subbaiah et al. 1998). Furthermore, NO is able to stimulate Ca^{2+} release from mitochondria (Richter 1997). In summary, NO reconfigures respiratory redox metabolism since it inhibits electron flow through the cytochrome pathway (maybe in favor of AOX) and stimulates NADH re-oxidation by alternative dehydrogenases. Also, as mentioned above, the involvement of haemoglobin in NO turn-over represents an alternative pathway (aside fermentation) for NAD^+ regeneration under low oxygen (Igamberdiev et al. 2010).

IV. Conclusion

Unlike animals, plants do not have a general system for oxygen delivery or direct oxygen sensing that triggers an organism-scale adaptive response. Despite this apparent weakness, plants exhibit a higher tolerance to low oxygen stress than animals. In fact, plants are able to survive much longer periods of oxygen shortage than animals: for example, up to several hours or several days of waterlogging- or submergence-induced hypoxia or anoxia, even in species not adapted to submersed soils or flooded areas. Recent transcriptomic, metabolomic and fluxomic

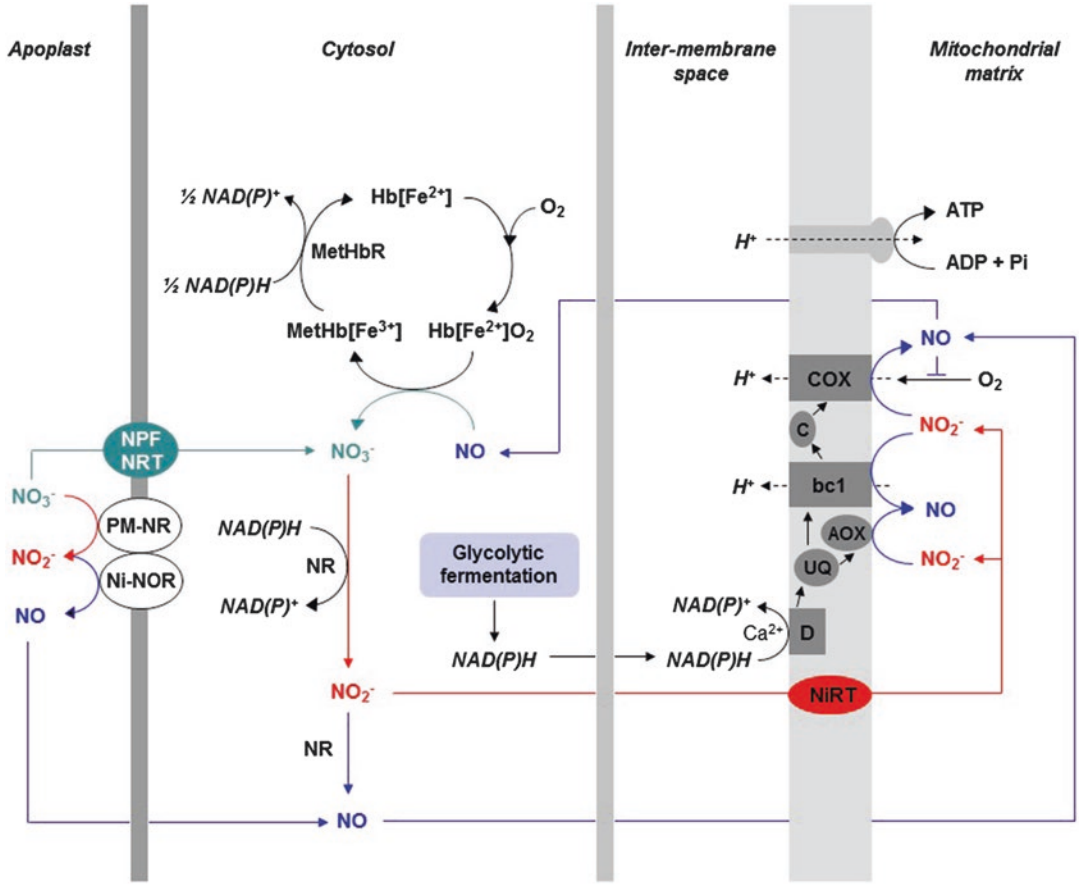

Fig. 10.3. **NO Production and its role in energy and redox metabolism through the haemoglobin-NO cycle under oxygen deficiency**. NO is produced by reductive pathways using nitrite as a substrate either by apoplastic NiNOR, cytosolic NR and/or mitochondrial electron transport chain. NO can be scavenged in the cytosol by oxyhaemoglobin (nsHb[Fe^{2+}]O_2), regenerating nitrate and methaemoglobin (nsHb[Fe^{3+}]) which is then reduced by methaemoglobin reductase (MetHbR). This nsHb-NO cycle contributes to cellular redox balance during hypoxia because it involves NAD(P)H oxidation events. As usual, ATP synthesis stems from the transmembrane electrochemical gradient generated by proton pumping along the mitochondrial electron transport chain. Scheme modified from Stoimenova et al. (2007). Abbreviations: *C*, cytochrome c; *D*, calcium-dependent mitochondrial NADH dehydrogenase; *NiRT*, nitrite transporter; *NPF*, nitrate transporter1/peptide transporter family; *NRT*; nitrate transporter; *UQ*, ubiquinone.

data have been instrumental in defining how C and N metabolisms are reorchestrated under low oxygen stress. In other words, the relatively good tolerance of plants to low oxygen seems to come from their ability to change metabolism. This involves enzyme regulations (TCA cycle, glycolysis, fermentation, and amino acid metabolism) triggered by indirect sensing of oxygen deficiency via alterations in, e.g. the redox poise (NADH/NAD^+ ratio), energy status (ATP content), reactive oxygen species (ROS), and nitric oxide (NO) production. Transcriptionally-controlled responses that depend on oxygen signaling based on the NERP proteolysis system are induced after O_2 cellular concen-

tration reaches a yet unknown threshold. Although not well characterized yet, the transcriptional response likely plays a role in both mid-term metabolic changes from aerobiosis to anaerobiosis and long-term adaptive effects such as hormone-dependent stem elongation and aerenchyma development.

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