



New Insights into the Metabolic and Molecular Mechanism of Plant Response to Anaerobiosis

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Abstract

Under anaerobic conditions, plants apply a wide spectrum of precise adaptive strategies responding to several critical challenges. The ability of efficiently sensing the oxygen presence demonstrates the existence of both direct and indirect ways of perception. The subsequent coordinate metabolic reassessment is currently under study. The complex molecular response implicates not only transcriptional and translational regulation of specific genes but also posttranscriptional and posttranslational regulatory mechanisms, each and all integrating the metabolic settings. Furthermore, the accumulation of typical metabolites during low oxygen stress condition is a key factor that suggests some critical topics in the regulation of metabolic pathways. Here, we summarize the main routes for adaptive behavior during oxygen depletion, from oxygen availability perception to recently discovered molecular mechanisms and metabolic adaptations.



1. INTRODUCTION

The Geologic Calendar is a scale in which the age of the Earth is assumed to be as long as 1 year. While *Homo sapiens* appears on the Earth in the last 4 min of this year, land plants cover the entire last month. This point of view may suggest the extent to which plants demonstrate an ability to recognize and face the wide variety of environmental conditions, even stressful and drastic, bypassing their main limit—being sessile—and colonizing the planet. Plant distinctive feature refers not only to the unavoidable necessity to face conditions without escape but also to the spread and unpredictability of seed dispersion, insomuch as second-generation plants can grow in environmental conditions that are completely different from those of their parent plants.

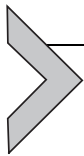
Both of these challenges led to the evolvement of a wide spectrum of adaptive strategies, insofar as a single plant may produce changes in metabolism in order to face an environmental state or a transient condition, inducing different metabolic responses depending on the degree of the stress conditions and involving the whole plant in a coordinated response. In particular, soil flooding—and thus anaerobiosis—is a stress condition that requires quick and precise sensing, well-coordinated signaling and an integrative response in order to bypass the stress without irreversibly impairing cell metabolism.

In responding to oxygen depletion at the molecular level, a main role has been observed for both transcriptional and translational regulations of specific genes, such as the hypoxia-related transcription factors (TFs)—in particular the family VII of the ERF (ethylene response factor) and heat shock transcription factors (HSFs), involved in oxygen sensing and stress response triggering (Licausi and Perata, 2009; Licausi et al., 2011; Pucciariello et al., 2012).

Furthermore, recent studies have highlighted the even higher complexity of this molecular response; posttranscriptional and posttranslational regulations also participate in the network of regulatory mechanisms (Mazzucotelli et al., 2008), integrating the modulation of metabolic settings. The metabolic and physiological reassessment induced by the lack of oxygen also depends on indirect sensing mechanisms involving different changes in cytosolic parameters and hormonal balances (Bailey-Serres et al., 2012). From a metabolic point of view, production of lactate and ethanol has been studied since 1974 when Davies and colleagues suggested the mechanisms regulating lactic and ethanolic fermentation.

More recently, studies of the accumulation of specific amino acids were found to belong to the adaptive response of the plant to the lack of oxygen. In particular, alanine and γ -aminobutyric acid (GABA) have been found to be the most biosynthesized amino acids during hypoxic conditions, with a role in maintaining the osmotic potential and in limiting the cytosolic acidification (Miyashita and Good, 2008). Succinate also accumulates, suggesting the last step of the noncircular TCA cycle active during oxygen depletion (Sweetlove et al., 2010), while the observed production of γ -hydroxybutyrate (GHB) during stress has been explained with the conversion of succinic semialdehyde derived from GABA-T by means of a specific reductase enzyme (Breitkreuz et al., 2003; Deleu et al., 2013; Renault et al., 2012).

Furthermore, metabolic adaptations also involve a mitochondrial role for nitrite, where it acts as an alternative electron acceptor in the electron transport chain, producing nitric oxide (Stoimenova et al., 2007) and contributing to the maintenance of mitochondrial activity and ATP synthesis during anoxic conditions (Igamberdiev et al., 2005; Stoimenova et al., 2007). Thus, plant strategies for adaptation to low oxygen conditions are defined by a complex coordination of molecular, metabolic, and physiological redefinitions, which allow the survival of plants even under strict low oxygen conditions.



2. CELLULAR OXYGEN STATUS PERCEPTION

2.1. Roots

Roots are very sophisticated organs; they ceaselessly maintain a highly complex interdependent relationship with biotic and abiotic components of soil. While root apparatus is strongly influenced by the environment, it also strongly influences the surrounding soil by means of exudates, water and oxygen uptake, and metabolic activities (Hinsinger et al., 2009). Furthermore, also among those processes operating inside roots, there is an interdependence that involves soil condition, soil condition sensing, and a subsequent redefinition of metabolic pathways, as seen, that is, for water uptake during anaerobic conditions (Tournaire-Roux et al., 2003). Actually, during flooding, plants act to redefine metabolic and molecular processes across the entire plant (Bailey-Serres and Voesenek, 2008; Christianson et al., 2010; Kreuzwieser et al., 2009). This broad rearrangement, which involves organs that are not directly suffering stress, suggests the presence of a systemic and incisive intraplant communication,

acting for the entire plant adaptation. Plant adaptation begins with the perception of stress and with the root taking on the task of perceiving the availability of oxygen. Root anatomy is composed of different zones, which are basically defined as root cap, which covers the root apices (Barlow, 2003), a meristematic zone, a transition zone, and a zone of rapid cell elongation (Baluška et al., 1990, 1996, 2001; Kieffer et al., 2009; Verbelen et al., 2006).

Focusing on the transition zone, we find that the cells of this zone have been shown to be the most intense zone for endocytic vesicle recycling and for auxin flux (Baluška et al., 2010; Sukumar et al., 2009). These cells show evidence of an electrical activity synchronized with auxin transport peaks (Masi et al., 2009) and an intense and complex hormonal network (Baluška et al., 2010). In order to sustain all of these processes, the transition zone is characterized by a very high intake of ATP and oxygen (Mancuso et al., 2000)—much higher than in the other root regions (Mancuso and Marras, 2006; Mugnai et al., 2011, 2012). Consequently, oxygen deprivation greatly affects the transition zone, which is the region of the root most sensitive to a lack of oxygen (Mugnai et al., 2012) and thus the probable point of departure of intercellular communication.

It is worth mentioning that oxygen availability sensing and intercellular signaling are a deeply intricate field of research, with many unclear points and no completely exhaustive answers. However, the key role of the transition zone in sensing and signaling oxygen deprivation may have been confirmed in 2012 when Mugnai and colleagues studied the effects of hypoxia selectively imposed on the root apex. The cells of the transition zone react to the stress by emitting an increased amount of nitric oxide, which seem to play an important role in inducing the entire root to have an acclimation response.

2.2. Multifactorial perspective of oxygen sensing and signaling

In order to favor plant survival during oxygen deprivation, the availability of oxygen can be perceived by both direct and indirect sensing, leading to a cascade of events belonging to the acclimation response (Bailey-Serres and Chang, 2005; Bailey-Serres et al., 2012). Some of the indirect mechanisms might involve the perception of the ratio of ATP and ADP, of the amounts of carbohydrates and pyruvate, of altered cytosolic pH and cytosolic calcium, and of the burst in reactive oxygen species (ROS) and nitrogen oxygen species (Bailey-Serres et al., 2012). These indicators of oxygen availability also participate in the response of other stress conditions, both

biotic and abiotic (Pucciariello and Perata, 2012). Some of them are not plant specific, but are important parameters for most living organisms.

Cytosolic-free calcium concentration is one of the most widespread signals among all living organisms. Calcium spikes are involved in the perception of many different stimuli or stresses; these spikes stimulate responses involved in gene expression, cell physiology, or metabolic pathways (Tuteja, 2009). During hypoxic and anoxic stresses, an increase in Ca^{2+} concentration has been observed in both maize and Arabidopsis cells (Sedbrook et al., 1996; Subbaiah et al., 2000). It has been speculated that this increase may be required for *Adh1* gene expression (Subbaiah et al., 2000).

Cytosolic pH change during anaerobiosis is a well-known effect since the lactic fermentation pathway prevails in early phases of anaerobic metabolism, resulting, first of all, in a fall of the cytoplasmic pH (Davies, 1980; Perata and Alpi, 1993). Curiously, it can be supposed that changes in pH values generated in the stressed cells of anaerobic roots might have signal roles in themselves. For example, cytosolic acidification is associated with the induction of ethanolic fermentation (Davies, 1980; Perata and Alpi, 1993), with a positive stimulus in the nitrate reductase activity, leading to an increased emission of NO (Dordas et al., 2004), and with a reduction in the hydraulic conductivity of the roots by affecting the activity of aquaporins (Bramley and Tyerman, 2010; Ehlert et al., 2009; Tournaire-Roux et al., 2003).

This malfunction in the aquaporins—and the subsequent decrease of root hydraulic conductance—results in a fall in leaf hydration and thus in an induced stomata closure in order to reduce water loss (Andersen et al., 1984; Else et al., 2001; Jackson et al., 2003). This cascade of events following a change in pH values in root anoxic cells supports the suggestion that pH may have a key role in sensing oxygen deficiency and triggering responses among the whole plant. Moreover, the acidification might diffuse through root cells until reaching the xylem sap, and then move and act as a messenger in itself across the plant (Jia and Davies, 2007).

Also nitric oxide has emerged as a very significant signal molecule and is involved in many different stresses (Leterrier et al., 2012; Sun et al., 2007; Wang and Yang, 2005). NO has been shown to have various key roles during anaerobiosis. NO is an alternative product of the altered mitochondrial metabolism, since nitrite becomes an intermediate electron acceptor and is converted back to nitrate by low oxygen-induced plant hemoglobin, becoming a fundamental step in the anaerobic switch of plant mitochondria (Igamberdiev and Hill, 2009). NO is implied in the hormone-induced response by means of synergic or counteractive interactions

(Leterrier et al., 2012). NO has also been suggested as a trigger for gene expression and as an inhibitor of mitochondrial enzymes (Blokhina and Fagerstedt, 2010).

As seen before, the cells of the transition zone in the root are those with the highest intake of ATP and oxygen; thus, it is hypothesized that these cells are highly sensitive to oxygen deficiency. Interestingly, an enhanced emission of NO has also been observed in these cells—an emission which it has been speculated is involved in the metabolic switch of these cells during stress associated with a decreased demand of oxygen (Mugnai et al., 2012).

2.2.1 Reactive oxygen species

Even if it does not seem intuitive, lack of oxygen is a stress condition which induces oxidative stress. Increased levels of ROS have been observed in both tolerant and sensitive species. Pigeon pea (*Cajanus cajan*), pea (*Pisum sativum*), and soybean (*Glycine max*) have shown markedly enhanced levels of ROS, in particular H₂O₂, when subjected to a lack of oxygen (Ershova et al., 2011; Kumutha et al., 2009). H₂O₂ accumulation in response to oxygen deprivation has also been detected in *Arabidopsis thaliana* seedlings (Baxter-Burrell et al., 2002), in roots of wheat (*Triticum aestivum*), and in two different species of apple trees (*Malus hupehensis* and *Malus toringoide*) (Bai et al., 2010; Blokhina et al., 2003).

Santosa et al. (2006) evaluated the oxidative stress of submerged rice, detecting ethane production as a product of membrane peroxidation. They observed that ethane emission was enhanced during submergence, as levels of O₂ fall to 1%, highlighting the ROS production and the subsequent cell damage during the decline in the availability of oxygen. ROS production is predominantly attributed to the pathway that involves the activity of a RHO-like, small G protein (ROP) and its interaction with a NAPH oxidase (NOX) localized in the plasma membrane (Sagi and Fluhr, 2006; Sairam et al., 2008; Wong et al., 2007).

NOX generates ROS in both animal and plants, and NOX-dependent superoxide production has been suggested as a key factor for adaptation during plant hypoxia (Sairam et al., 2008). In fact, the interaction between ROP and NOX promotes a cascade of events that induces an enhanced calcium influx, an increase in the H₂O₂ levels, and a subsequent switch to anaerobic fermentation and other stress adaptive pathways (Baxter-Burrell et al., 2002; Demidchik and Maathuis, 2007; Pucciariello et al., 2012).

A mitochondrial role in ROS production has also been hypothesized. In 2012, Chang and colleagues studied ROS production in *Arabidopsis* seedlings when the electron transport chain was inhibited at the sites of

complexes III and IV (Chang et al., 2012). Experiments provided evidence about the transient activation of mitogen-activated protein kinases and the ubisemiquinone accumulation, with an associated increase in ROS production. Hydrogen peroxide, being an uncharged molecule, can easily diffuse through the mitochondrial membrane, reaching the cytosol and acting as signal of the stress condition (Han et al., 2003; Turrens, 2003). As suggested by these studies, there is evidence of a ROS role in the signaling mechanisms in plants as triggers for the stress response and as molecules involved in the cell-to-cell communication (Mittler et al., 2011). Observing the characteristics of ROS, they seem to be perfectly fitting as having a role as signal molecules. The quick production, the ability to induce specific responses, and the rapid degradation are key features for the effective signal molecule (Pauly et al., 2006).

During oxygen depletion, a set of genes related to the balance between ROS production and ROS scavenging is newly regulated, thus coordinating the ROS-dependent response (Pucciariello et al., 2012). Moreover, the lack of oxygen implies ROS-dependent TFs and heat-shock proteins (HSPs), strictly related to the presence of ROS. The involvement of all of these ROS and ROS-dependent factors during oxygen shortage also suggests a possible signaling role of ROS in themselves (Pucciariello et al., 2012). Indeed, ROS molecules have been demonstrated to have an importance in cell-to-cell signaling, following mechanisms which seem to be common between anoxic and heat stresses, since the oxidative burst belongs to both these stress conditions (Banti et al., 2010). In particular, H₂O₂ involvement has been reported for the activation of redox-sensitive TFs, such as heat shock transcription factors (HSFs) in different stress conditions involving an oxidative stress (Pucciariello et al., 2012). Hydrogen peroxide also has been shown to be involved in the low oxygen, stress-induced formation of lysigenous aerenchyma in Arabidopsis plants (Muhlenbock et al., 2007) and in the programmed cell death induced by abiotic stress (De Pinto et al., 2012). Moreover, Arabidopsis plantlets pretreated with H₂O₂ demonstrated enhanced tolerance to anoxia (Banti et al., 2010).

2.2.2 Hormonal involvement

Plant hormonal balances involve, during oxygen depletion, the rearrangement of ethylene, abscisic acid, and gibberellin (GA) levels (Yemelyanov and Shishova, 2012). Their coordinated effect is implied in the adaptive pathways of both tolerant and sensitive plants, mediating molecular and physiological responses (Bailey-Serres and Voesenek, 2008; Kende et al., 1998; Peeters et al., 2002).

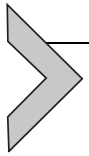
Ethylene production seems to be the first hormonal regulation during low oxygen conditions, becoming the trigger for the escape strategy in tolerant species. Ethylene stimulates the growth rate of both the shoot and the leaf (Jackson, 2008), as well as the activity of specific, ethylene-dependent enzymes involved in cell loosening (Bragina et al., 2001, 2003). Ethylene plays an important role in the aerenchyma formation (Sairam et al., 2008), as demonstrated in hypoxic roots of maize, where it has been confirmed by means of exogenous application of ethylene antagonists, which were shown to exert an inhibitory effect on its development (Drew et al., 1981; Jackson et al., 1985; Konings, 1982). For most of the plants studied, ethylene production during flooding was shown to induce hyponastic leaf growth (Ridge, 1987; Voesenek and Blom, 1989). Moreover, ethylene production may have an inhibitory effect on physiological parameters during hypoxic stress, as seen in tomato plants where an increased ethylene/auxin ratio affected the formation of adventitious roots (Vidoz et al., 2010).

Ethylene also regulates the induction of hypoxia-related genes, even if only 10% of the so-called ERFs require ethylene to be regulated (Nakano et al., 2006). These ethylene-dependent ERFs seem to be fundamental for sensing oxygen, as well as for the beginning of an adaptive response (Voesenek and Sasidharan, 2013). The ethylene-inducible ERFs belonging to the family VII have been thoroughly studied in rice, since they can act as mediators for both quiescent and escape strategies. Particularly during the quiescent strategy, the group VII ERF Submergence 1A (Sub1A)-1 hinders underwater growth, inhibiting the induction of those genes codifying for proteins involved in starch breakdown and cell wall loosening (Fukao et al., 2006) but also favoring the activity of specific repressors of GA-induced growth processes (Fukao and Bailey-Serres, 2008).

In contrast, during the escape strategy, the Snorkel locus encodes for two ERF VII groups, Snorkel 1 (SK1) and Snorkel 2 (SK2), which promote fast stem elongation in order to reach the water level (Fukao and Xiong, 2013; Hattori et al., 2009).

Coordination among different hormones is clear in submerged organs of rice, where the induction of ethylene promotes a subsequent regulation of the balance between aminobutyric acid (ABA) and GAs, mediating cell elongation (Bailey-Serres et al., 2012). Reduced levels of GAs during oxygen deprivation have also been observed in *Rumex palustris* and *Rumex acetosa*. Reduced GAs, with a simultaneous increase in ABA levels, coordinate the reduced elongation (Benschop et al., 2005; Chen et al., 2010). It has been suggested that during waterlogging, both GAs and cytokinins may have

a role in root-to-shoot signaling by promoting stomatal opening, in antagonism with the ABA effect (Kumar et al., 2004; Pospíšilová, 2003). It has also been observed that NO levels rise during oxygen deprivation while GA levels decrease, suggesting a role in stomatal closure (Christianson et al., 2010).



3. MOLECULAR MECHANISMS IN ANAEROBIC RESPONSE

During hypoxic and anoxic stress, plant adaptive strategies have been divided into two broad groups: low oxygen quiescence syndrome (LOQS) and low oxygen escape syndrome (LOES) (Colmer and Voesenek, 2009). However, some responses have been shown to be conserved in all flooding-adapted plants, independently from the strategy. Since oxygen availability becomes low during submergence, plants following the LOQS strategy reduce or repress stem elongation and keep metabolic pathways in a quiescent state in order to limit energy-consuming processes and save substrate supply for the desubmergence phase. In contrast, plants responding with an LOES strategy show the ability to tolerate longer term anoxic stresses, investing in the faster elongation of submerged stems, petioles, and leaves, and thus reaching the water surface to be back in contact with air (Colmer and Voesenek, 2009; Pucciariello and Perata, 2012). Curiously, it has been proved that there is not a sharp distinction between the two strategies, since some tolerant species have the ability to “choose” the one or the other strategy depending on the seriousness of stress conditions, showing a very sophisticated stimuli response (e.g., see *Oryza sativa* spp. indica, Bailey-Serres et al., 2012). This basic differentiation in possible responses to oxygen deprivation is also shown in the numbers of genes selectively expressed during the stress, which present significant differences between flooding-sensitive plants and flooding-tolerant ones. During hypoxic stress, a flooding-tolerant plant, such as poplar, shows altered expression levels for more than 5000 genes. In contrast, the flooding-sensitive *Arabidopsis* plants show a change in the transcript abundance of about 150 genes involved in the stress response (Klok et al., 2002; Liu et al., 2005).

In particular, *Arabidopsis* and rice are the most thoroughly studied plants in their response to hypoxia and anoxia, and today, many microarray data sets provide a notable amount of information (Banti et al., 2010; Branco-Price et al., 2005; Jung et al., 2010; Lasanthi-Kudahettige et al., 2007; Lee et al., 2011; Licausi et al., 2010; Liu et al., 2005; Loreti et al., 2005; Mustroph et al., 2010; van Dongen et al., 2008). Furthermore, even in

the same plant, upregulation or downregulation of specific genes seems to depend strongly on the degree of the stress condition (i.e., from the beginning of hypoxia to a strict anoxia), involving both common and different genes for each stage of oxygen deprivation, as seen for *Arabidopsis* (Pucciariello et al., 2012). It has also been noted that there are genes whose regulation occurs throughout the entire plant, as well as specific genes differently regulated depending on the organ of the plant (Pucciariello and Perata, 2012).

In spite of these variances, a common trait for sensitive and tolerant species, as well as for different stress levels, is that a high percentage of genes involved in the stress response is composed of TFs. TFs play the part of the last regulatory step in the signal cascade, starting from the perception of the environmental change and advancing toward the metabolic response. Several hypoxia-responsive TFs have been studied, that is, the MYB (myeloblastosis), the ATAF (*Arabidopsis* transcription activation factor), the PHD (plant homeodomain), and the ERF families (Bond et al., 2009; Christianson et al., 2009; Hoeren et al., 1998; Licausi et al., 2010). But in any case, no single TF seemed to be the only one regulating the stress response. This finding suggests the existence of a more complex transcriptional network with coordinated transcriptional regulators which contribute to the stress adaptation in the molecular response to a lack of oxygen (Kreuzwieser et al., 2009; Licausi et al., 2010).

3.1. ERF VII transcription factors

3.1.1 *ERF VII as key players in anaerobic adaptation*

Among the TFs studied, particular attention has been directed to the family VII of the ethylene response factor (ERF VII) involved in stress adaptation responding to increased ethylene production (Nakano et al., 2006). Both LOES and LOQS mechanisms involve genes belonging to this family (Pucciariello and Perata, 2012), even if there are consistent differences among species. For example, ERF VII members identified in monocots such as rice and maize are double or more in respect to the numbers of those found in the dicots studied (Licausi et al., 2010; Zhang et al., 2008).

In mature rice plants using the quiescent strategy involved in the flooding response, the ERF Sub1A gene has been demonstrated to play important roles (Fukao et al., 2006; Xu et al., 2006). Rice SUB1A has a primary role related to the presence of ADH, improving the fermentative metabolism (Fukao et al., 2006). But SUB1A also induces the repression of those genes involved in sucrose degradation by means of sucrose synthase (Fukao et al.,

2006), and the inhibition of the GA-mediated underwater plant elongation, limiting cell expansion (Fukao and Bailey-Serres, 2008).

In particular, during the quiescent strategy, the submergence-induced allele Sub1A-1 activates two specific genes: the slender-rice 1 (SLR1) and the slender-rice-like 1 (SLRL1). These genes inhibit GA-induced plant elongation leading to the preservation of the energy reserve (Bailey-Serres and Voesenek, 2008; Pucciariello and Perata, 2012). In contrast, in rice plants utilizing the escape strategy, underwater plant elongation is induced by two different genes also belonging to the ERF family VII: SK1 and SK2, which have a role in promoting fast elongation, in order to reestablish gas exchange once the plant has reached the water surface (Hattori et al., 2009).

Moreover, Arabidopsis plants subjected to low oxygen conditions have shown an increased regulation of genes belonging to the ERF family VII, in particular five members: HRE1, HRE2, RAP2.2, RAP2.3, and RAP2.12 (Licausi et al., 2010).

The hypoxia-responsive ERF (HRE)1 and the HRE2 are both localized in the nucleus, where, as suggested by the literature, they may have the role of positively regulating gene transcription (Licausi et al., 2010; Xu et al., 2007). Both HRE1 and HRE2 are not directly induced by the increase in ethylene production, although their response to anaerobic stress quickly emerges (van Dongen et al., 2009). Experiments with transgenic plants confirmed the importance of these genes. Overexpressing HRE1 and HRE2 plants enhance their tolerance to anoxia, while the suppression of both HRE1 and HRE2 induces an increased sensitivity, lowering the expression of many hypoxic-related genes (Pucciariello and Perata, 2012). Thus, Arabidopsis plants lacking oxygen clearly show an increased expression of ERF VII genes and an increased expression of hypoxic genes, with a subsequent enhanced low oxygen tolerance (Hinz et al., 2010). Despite that, only plants overexpressing HRE1 show an intensified hypoxic induction of the anaerobic genes ADH, SUS1, and SUS4 and an upregulation of ADH under aerobic conditions (Licausi et al., 2010).

RAP2.2 is constitutively expressed at very high levels in roots and has a role in the induction of genes related to the sucrose metabolism and the fermentation pathways (Hinz et al., 2010). Its distribution across the plant reflects the dissemination of anaerobiosis-related genes such as ADH1, LDH1 (Dolferus et al., 2008), and AlaAT1 (Miyashita et al., 2007). Even if RAP2.2 induction under hypoxia seems not to be drastic (Hinz et al., 2010), transgenic plants overexpressing RAP2.2 showed an improved

tolerance to hypoxic stress, whereas the knockouts of the gene caused a decreased survival rate (Hinz et al., 2010).

However, overexpression of RAP2.2 did not affect the expression of ADH gene or any other anaerobic gene in air (Welsch et al., 2007). Also RAP2.12, whose role was suggested to be in the upregulation of the ADH1 expression and ADH activity (Papdi et al., 2008), did not show an increased gene expression in *Arabidopsis* seedlings subjected to hypoxia (Licausi et al., 2010). RAP2.3 is known to confer resistance to H₂O₂ and to positively regulate many defense genes (Ogawa et al., 2005; Papdi et al., 2008). Like RAP2.2 and RAP2.12, the expression of RAP2.3 was also unaffected by hypoxia (Licausi et al., 2010).

3.1.2 ERF VII as key players in oxygen sensing

In the past, many different hypothetical oxygen-sensing mechanisms were studied. In 2007, there emerged a possible role for prolyl hydroxylase enzymes, whose transcription seemed to increase during the oxygen deficit in *A. thaliana* and in rice (Lasanthi-Kudahettige et al., 2007; Vlad et al., 2007). In mammals, prolyl hydroxylase is an enzyme that works consuming O₂, implicated in the degradation of a TF subunit involved in oxygen-deficiency acclimation (hypoxia-inducible factor 1 α —HIF1 α). The enzyme activity is inhibited in oxygen-deficiency conditions and thus TFs related to the stress are not prevented anymore. Therefore, in mammals, prolyl hydroxylase perceives the weakening of O₂ and behaves as a direct sensor of hypoxia (Guzy and Schumacker, 2006).

In spite of these theories, no HIF1 α has been found in plants (Bailey-Serres et al., 2012). More recently, new, promising studies have shown a molecular response to hypoxia that involves the subgroup VII of the ERF and their posttranslational regulation (Licausi et al., 2011, 2013). An important role for oxygen availability sensing and hypoxia response had already been assigned to the hypoxia-related ERFs, since many different studies showed a strong relationship among oxygen conditions, induction of ERFVII genes, and subsequent reassessment of metabolic routes (Hinz et al., 2010; Licausi et al., 2010, 2011; Papdi et al., 2008).

Subsequently, a cascade of findings supported the hypothesis that ERF TFs belonging to the group VII subfamily are also hypoxia sensors in plants, suggesting that the oxygen perception involves their posttranslational regulation by the ubiquitin-dependent N-end rule pathway (NREP) for protein degradation (Licausi et al., 2010, 2011). In particular, RAP2.12, a member of the ERF VII, has been demonstrated to play a fundamental role in the

trigger of the anaerobic response (Licausi et al., 2011). In 2008, Papdi and colleagues suggested that RAP2.12 could bind the ADH1 promoter. A posttranscriptional or posttranslational regulation of RAP2.12 emerged when mutants overexpressing RAP2.12 revealed an increase in the expression of those hypoxia-responsive genes only in those plants suffering hypoxic conditions, suggesting that normoxic conditions inhibit the action of RAP2.12 (Licausi et al., 2011; Sasidharan and Mustroph, 2011). A conserved amino-terminal sequence, which includes the Met-Cys initiating motif (MCGGAI/L), is shared by the hypoxia-responsive factors HRE1, HRE2, RAP2.2, and RAP2.12 (Licausi et al., 2011, 2013). This conserved domain is a peculiar motif, a perfect target for the N-end rule degradation mechanism (Gibbs et al., 2011; Licausi et al., 2011), which is an oxygen-dependent, posttranslational pathway, able to lead the protein degradation under aerobic conditions. Thus, during hypoxic conditions, the NERP is inhibited, and RAP2.12 is not degraded and moves to the nucleus where it accumulates, inducing the expression of those genes involved into hypoxia acclimation (Fig. 5.1; Licausi, 2011; Licausi et al., 2011).

Confirming this hypothesis, it has been observed that during normoxic conditions RAP2.12 can be recruited by the membrane ACBPs (Acyl-CoA binding proteins) and, being temporarily sequestered and protected from degradation, then can undergo degradation by means of the NERP. In contrast, during hypoxia, RAP2.12 dissociates from the membrane to move into the nucleus (Licausi et al., 2013).

3.2. Nontranscriptional regulation in low oxygen sensing and response

3.2.1 Role of polyribosomes: Example of posttranscriptional regulation

It is worthwhile to highlight the role of the differential mRNA translation, in particular, for those mRNAs associated with large polyribosomes (Branco-Price et al., 2005). It has been suggested an optimization of the mRNAs selective translation during stress, in order to grant an efficient translation of the hypoxia-induced mRNAs (Branco-Price et al., 2008). Interestingly, the posttranscriptional regulation also grants an increase in the translation of those mRNAs which encode enzymes that participate in the anaerobic production of ATP (Branco-Price et al., 2008), even if there is no alteration in the abundance of mRNA transcript. Moreover, during the stress, some mRNAs do not undergo translation, since they can be stabilized or degraded once sequestered into cytosolic messenger ribonucleoprotein complexes (mRNPs) (Branco-Price et al., 2008; Hoyle et al., 2007;

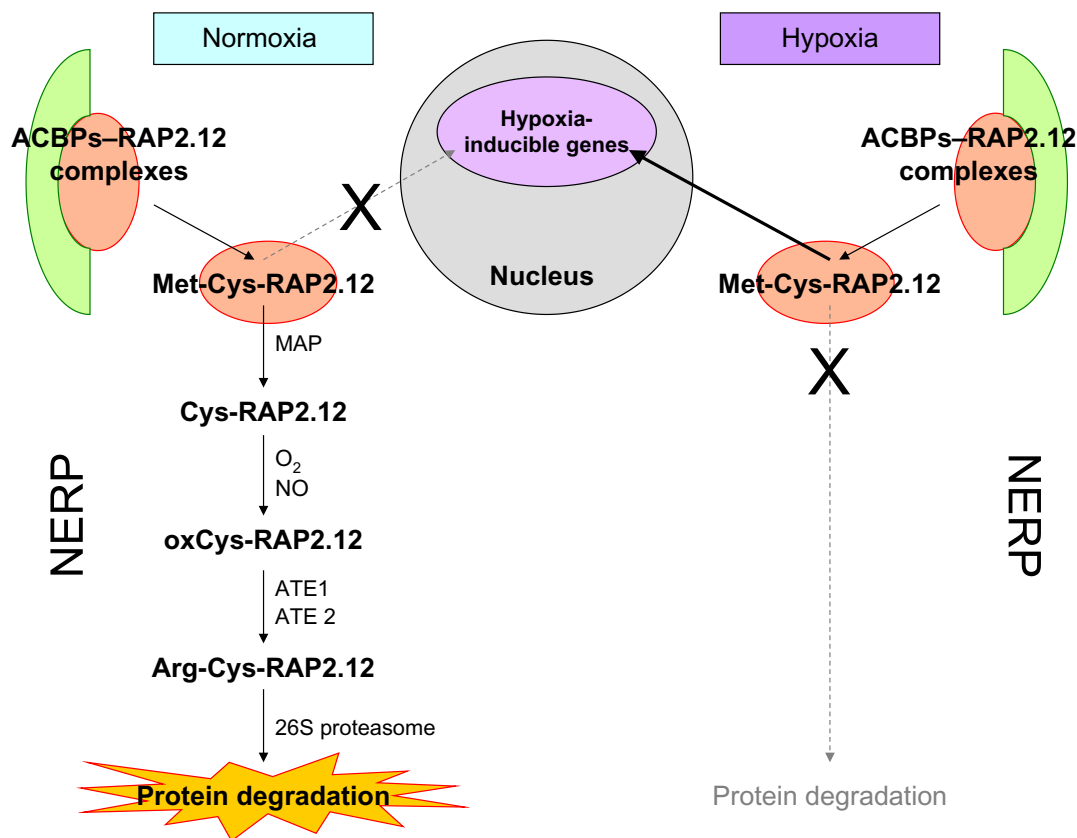


Figure 5.1 The N-end rule pathway during normoxic and hypoxic conditions. During normoxic conditions, the RAP2.12 transcription factor bound to ACBPs (acyl-CoA binding protein 1 and 2) is a target for proteasomal degradation following the N-end rule pathway (NERP). N-terminal Met cleavage catalyzed by MAP leaves the cysteine residue exposed to oxidation. Oxidized cysteine becomes then the substrate for the addition of an arginine and the subsequent degradation of the polypeptide. During oxygen deficiency, NERP is inhibited and the protein can move to the nucleus, where it triggers hypoxia-related signaling cascade enhancing plant survival. ACBPs, acyl-CoA binding proteins 1 and 2; ATE, arginyl tRNA transferase; MAP, methionine-amino peptidase; NERP, N-end rule pathway.

Parker and Sheth, 2007). This mRNP–mRNA complex allows the preservation of those mRNAs that under stress conditions are not translated, delaying their degradation (Arru and Fornaciari, 2010).

To simplify, during low oxygen conditions, a loss of correspondence between gene transcription and enzyme synthesis can be observed. Some mRNAs are sequestered by mRNPs so as not to be translated, but also not degraded. In contrast, those mRNAs useful to overcome the stress may have an unchanged expression level associated with enhanced activity of the related enzyme. Where the abundance of mRNA is unchanged, the recruitment by polyribosomes can involve a significant advantage.

The population of mRNA involved in this posttranscriptional regulation represents a considerable number of genes which are important for stress-induced metabolism (Branco-Price et al., 2005). In particular, concerning the posttranscriptional regulation of TFs, it has been reported that HRE1 and HRE2 mRNAs under hypoxia are associated with polyribosomes (Branco-Price et al., 2005, 2008), highlighting once more how transcription and translation coordinate at multiple levels. Concerning the discrepancy between gene expression variance and protein activity of the TFs RAP2.2 and RAP2.12, the issue may have a different explanation, as elucidated recently by Licausi et al. (2013).

3.2.2 N-end rule pathway: Example of posttranslational regulation

The NERP is a proteolytic mechanism which depends on the interaction of the proteasome with its target, called N-degron, which is an N-terminal residue followed by a specific sequence of amino acids (Tasaki et al., 2012). This pathway belongs to the ubiquitin–proteasome system, which determines protein stability in eukaryotes participating in many different cellular and developmental processes (Graciet and Wellmer, 2010). In particular, for plants, the NERP is involved in leaf senescence and seed germination as well as in shoot and leaf development (Graciet and Wellmer, 2010; Holman et al., 2009; Yoshida et al., 2002). In plants, the homeostatic response to hypoxia depends on the halt of the NERP related to specific ERF VII TFs. In normoxic conditions, the same proteins undergo proteolysis by means of the NERP through a mechanism which leads to a proteasome target beginning with the modification of the ERF VII tertiary destabilizing residue.

The definition of this residue depends on the classification of the N-terminal amino acids, which includes two main categories, depending on the stability conferred to the protein: stabilizing N-terminal residues and destabilizing N-terminal residues.

The stabilizing N-terminal residues assure the safety of the protein against the NERP, stunting the protein alteration and subsequent degradation. In contrast, destabilizing N-terminal residues are good targets for ubiquitin-dependent protein degradation (Varshavsky, 1997). Moreover, destabilizing residues can be further divided into primary, secondary, and tertiary destabilizing amino acids, depending on the protein modification progress status (Licausi et al., 2013; Varshavsky, 1997).

Primary, secondary, and tertiary destabilizing amino-terminal residues of amino acidic sequences may be modified through different ways in order to allow protein degradation (Tasaki et al., 2012). Tertiary destabilizing

residues are those which require two steps of modification in order to become N-degrons, passing through the intermediate secondary status. The enzymatically catalyzed covalent modifications of tertiary destabilizing amino acids, such as deamidation or oxidation, alter the N-terminal residue leading to the following step: the secondary destabilizing residues. Thus, the addition of an Arg residue by means of arginyl tRNA transferases (ATEs), allows the formation of the primary destabilizing residue, which is the N-terminal proteasome target, therefore the N-degron (Fig. 5.2) (Licausi et al., 2013).

Depending on the oxygen availability status, some ERF VII TFs, for instance RAP2.12, are targets for proteasomal degradation following the NERP until oxygen is available. As shown in Fig. 5.1, the conserved amino-terminal sequence, which includes the MCGGAI/L, is the residue involved in the enzymatically catalyzed reactions. First, methionine-amino peptidase (MAP) mediates the Met cleavage (Liao et al., 2004), leaving an exposed cysteine residue, subjected to oxidation due to oxygen and nitric oxide. Consequently, oxidized cysteine becomes the substrate for the addition of an arginine and the subsequent degradation of the polypeptide (Bailey-Serres et al., 2012; Licausi et al., 2013). In contrast, during oxygen deficiency, NERP is inhibited and the protein moves to the nucleus, where it induces expressions of core hypoxia-related genes such as ADH1, PDC1, and SUS4, thus enhancing plant survival (Gibbs et al., 2011).

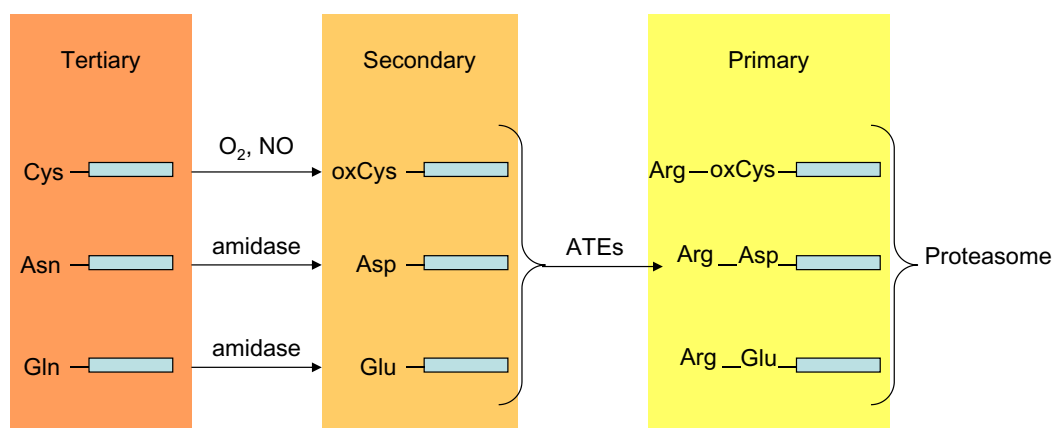


Figure 5.2 Schematic representation of oxidative and enzymatic reactions of tertiary, secondary, and primary destabilizing residues in the N-end rule pathway. Tertiary destabilizing residue can be degraded by means of deamidation or oxidation, forming an altered N-terminal residue. This secondary destabilizing residue is subjected to the addition of an Arg residue by means of arginyl tRNA transferases (ATEs). The subsequent primary destabilizing residue is then the N-terminal proteasome target.

3.3. Heat stress and oxygen-deprivation stress responses: Converging strategies

The main difference between hypoxia and anoxia response might be the greater or lesser involvement of HSFs and HSPs in the stress response. When the lack of oxygen reaches values ascribable to the anoxic condition, an overlap has been observed between heat stress-induced response and anoxia-induced response (Banti et al., 2010; Pucciariello et al., 2012). This convergence seems to be imputable to the higher amount of ROS, in particular H₂O₂, during each of these stresses (Banti et al., 2010). The increase in HSPs transcripts during anaerobic stress is widely conserved among different plant species (Mustroph et al., 2010; Vandenbroucke et al., 2008). Members of the HSFs are believed to sense indirectly the oxygen status, acting as H₂O₂ molecular sensors (Miller and Mittler, 2006) and modulating a complex response which confers improved tolerance to anoxia (Bailey-Serres et al., 2012). Anoxic stress imposed on Arabidopsis seedling induces the activation of HSPs-related genes (Banti et al., 2010, 2013), whose preinduction by means of heating has been demonstrated to confer a better tolerance to a later-imposed anoxia (Banti et al., 2010).

Transgenic plants of Arabidopsis overexpressing the TF HsfA2 showed an increased expression of small HSPs and antioxidant-related genes and were demonstrated to better tolerate anoxia (Hinz et al., 2010; Nishizawa et al., 2006; Ogawa et al., 2007; Schramm et al., 2006). Curiously, this TF does not seem to be related to the switch to the anaerobic metabolism (Banti et al., 2010). Also at the sensing level, there is no correlation between the anoxic-induced, heat-shock, overlapping response and the hypoxic-induced, posttranslational, regulation response (Licausi et al., 2011; Sweetlove et al., 2010).

This lack of correlation may suggest that there are at least two main ways to follow to overcome oxygen-deficiency stress. A first pathway begins with a sensing of the oxygen status by means of posttranslational regulation of specific ERFs and involves the modulation of several genes among which are those related to anaerobic metabolism. A second pathway depends on a ROS-dependent sensing mechanism and involves HSFs and HSPs, increasing anoxia tolerance through other defense strategies. For example, in Arabidopsis plants, the expression level of ascorbate peroxidases gene depends on the activity of the HSFs (Panchuk et al., 2002), suggesting that these TFs are involved in the oxidative stress regulation by means of antioxidant activity modulation.



4. METABOLIC ADAPTATIONS

Anoxic stress induces rapid and severe metabolic and molecular adaptations in order to confront the fall in ATP production, due to the lack of oxygen as the final acceptor of electrons in oxidative phosphorylation, the fall in cytosolic pH, and the imbalance in osmotic potential. There is evidence about changes in enzyme composition in the cytosol and in mitochondria (Couee et al., 1992; Igamberdiev and Hill, 2009; Igamberdiev et al., 2004; Miyashita and Good, 2008), mainly related to the fermentation pathways, to the GABA shunt, to the switch of the TCA cycle to a non-circular TCA flux, and to the hemoglobin/nitrate cycle.

In order to elucidate these substantial metabolic changes, it would be strategic to retrace pathways, cycles, and fluxes beginning from the hypothetically induced metabolites accumulation and turnover, and then defining the events in plant cells during anaerobiosis. During root anaerobiosis, several metabolites accumulate—especially GABA and alanine (Miyashita and Good, 2008; Reggiani et al., 2000) but also GHB (Nakamura et al., 2012) and succinate (Bailey-Serres et al., 2012). Like the well-known lactate and ethanol synthesized during fermentation, their production and accumulation in plant tissues are a challenge for survival during the depletion of oxygen availability in the soil.

4.1. GABA accumulation: “GABA shunt”

GABA metabolism during anoxia mainly involves a short pathway of three enzymes moving from glutamate and generating succinate, bypassing steps of the tricarboxylic cycle, and then being called “GABA shunt” (Bouché and Fromm, 2004). The steps of this metabolic pathway begin with the cytosolic enzyme glutamate decarboxylase (GAD) which converts glutamate into GABA, then the mitochondrial enzyme GABA transaminase (GABA-T) converts GABA into succinyl semialdehyde, and finally the enzyme succinic semialdehyde dehydrogenase leads to the biosynthesis of succinate (Bouché and Fromm, 2004). Interestingly, Miyashita and Good, in 2008, demonstrated that an inhibition of the GAD and GABA-T enzymes (and thus a defect in the GABA shunt) affected also the accumulation of alanine in *Arabidopsis* roots. In Fig. 5.3, the possible ways for alanine production, both independent and dependent from the GABA-shunt pathway, are shown.

In their 2009 review, Igamberdiev and Hill noted that alanine begins to accumulate earlier than GABA. The previous year, Miyashita and Good

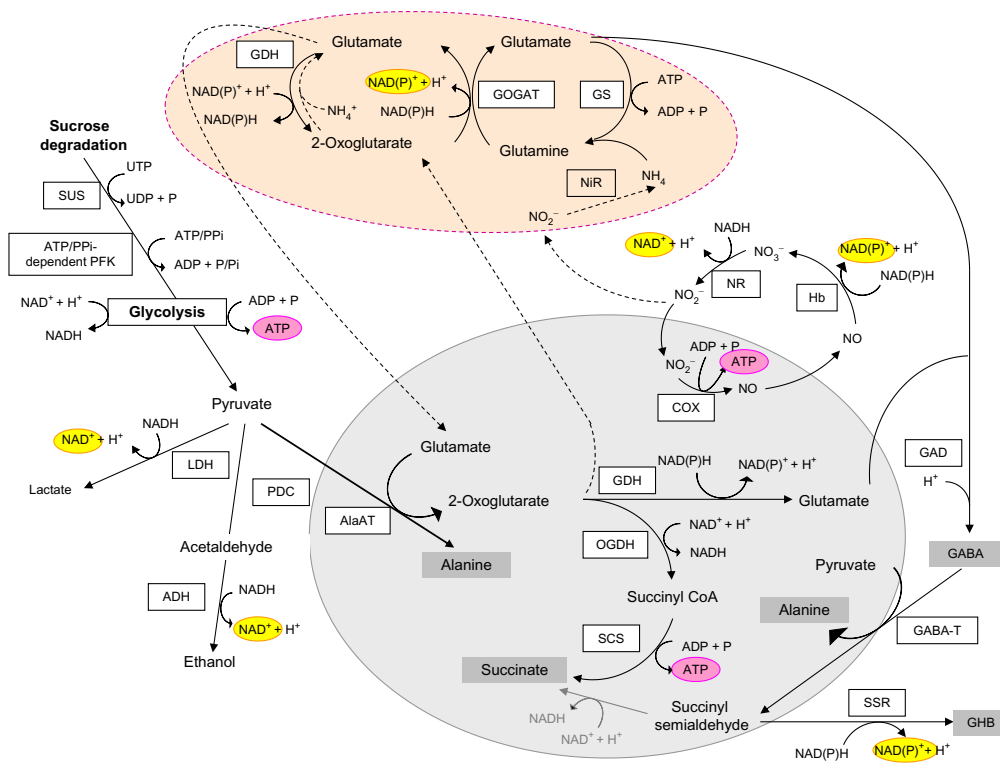


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demonstrated that in *Arabidopsis* roots, the inhibition of GAD and GABA-T was impaired (beyond the accumulation of GABA throughout the entire time of the experiment). Their study also saw the impairment of the accumulation of alanine in the second phase of the hypoxic experiment (beyond 4 h of hypoxia), while the initial rapid accumulation of alanine was not influenced by the GABA-shunt pathway. During oxygen-deficiency stress, a significant amount of succinic semialdehyde derived from the GABA-T activity is converted to GHB by means of the enzyme succinic semialdehyde reductase (SSR) (Breitkreuz et al., 2003; Deleu et al., 2013), which utilizes NADH contributing to redox regulation and providing NAD^+ , necessary for the glycolysis sustenance.

This raises the question: Which role might have GABA in the survival strategy of an anoxic cell? It is known that GABA has an important role contrasting the decrease in cytosolic pH and osmotic potential, contributing to the regulation of C:N balance, and hypothetically protecting cells from the oxidative stress (Banti et al., 2013; Bouché and Fromm, 2004; Bouché et al., 2003; Igamberdiev and Hill, 2009). In particular, it has been demonstrated (Shelp et al., 1999; Snedden et al., 1995, 1996) that GABA accumulates at low pH, GAD consumes a proton, and its activity is induced by cytosolic acidification. Moreover, accumulation of GABA is an adaptive strategy to prevent carbon loss and maintain osmotic potential, which falls as a consequence of the faster anaerobic carbohydrate consumption (Miyashita and Good, 2008; Reggiani et al., 2000).

Figure 5.3—Cont'd Metabolic reassessment during anaerobic stress. The alternative pathway responds to the energy crisis increasing ATP production and regenerating oxidizing potential. The scheme reports the main metabolic routes: enhanced sucrose degradation by means of sucrose synthase (SUS), sustained glycolysis by means of NAD^+ regeneration, fermentation, succinate accumulation, alanine and 2-oxoglutarate (2-OG) shunt and γ -aminobutyric acid (GABA) shunt, γ -hydroxybutyrate (GHB) accumulation, and enhanced GS-GOGAT cycle activity. Continuous lines indicate the followed paths during the stress; dashed lines indicate proposed active paths during the stress. Metabolites that accumulate during the stress (alanine, succinate, GABA, GHB) are written inside a dark grey squared box; newly synthesized ATP, newly regenerated NAD^+ and NAD(P)^+ are written inside elliptical-shaped boxes. 2-OG, 2-oxoglutarate; ADH, alcohol dehydrogenase; AlaAT, alanine aminotransferase; COX, cyclooxygenase; Hb, hemoglobin; GABA, γ -aminobutyric acid; GABA-T, GABA transaminase; GAD, glutamic acid decarboxylase; GDH, glutamate dehydrogenase; GHB, γ -hydroxybutyrate; GS-GOGAT, glutamate synthase cycle; LDH, lactate dehydrogenase; NiR, nitrite reductase; NR, nitrate reductase; OGDH, 2-oxoglutarate dehydrogenase; PDC, pyruvate decarboxylase; SCS, succinyl CoA ligase; SSR, succinic semialdehyde reductase; SUS, sucrose synthase.

As shown in Fig. 5.3, the GABA shunt might involve, indirectly, other metabolic pathways such as the GS/GOGAT (Bouché and Fromm, 2004; Reggiani et al., 2000).

In 2000, Reggiani and colleagues demonstrated that the use of inhibitors of the GS and GOGAT reactions significantly reduced the accumulation of alanine and GABA, proving that the GS/GOGAT cycle is necessary to assimilate nitrogen and produce those glutamate molecules that are the first step in the GABA-shunt reactions (Bouché and Fromm, 2004; Reggiani et al., 2000).

4.2. Alanine accumulation

As shown in Fig. 5.3, under anaerobic conditions, alanine production follows two possible different ways. Pyruvate can be converted to alanine by the transfer of an amino group derived from glutamate, which generates 2-oxoglutarate (2-OG) as a coproduct (Banti et al., 2013; Branco-Price et al., 2008). This reaction involves the Ala aminotransferase enzyme (AlaAT) since this enzyme is quickly induced under hypoxic conditions (Miyashita and Good, 2008; Muench and Good, 1994). However, in 2007 Miyashita et al. demonstrated that in anaerobic roots of *Arabidopsis*, AlaAT activity was not required for the alanine accumulation, suggesting the involvement of other routes which lead to alanine accumulation. In 2008, Miyashita and Good investigated the role of the GABA shunt in alanine accumulation, finding a relationship between the GABA-T activity and the alanine accumulation in hypoxic roots of *Arabidopsis*, in particular after the first 4 h from the start of the stress.

Alanine accumulation is known to be not toxic for the cells (Miyashita et al., 2007; Ricoult et al., 2005), to have a role in storing carbon and nitrogen, preventing their loss (Ricoult et al., 2005; Sato et al., 2002), and maintaining the osmotic potential (Miyashita and Good, 2008; Reggiani et al., 2000). Moreover, alanine production involves the generation of 2-OG (by means of the AlaAT activity) which can enter the partial (non-circular) TCA flux and proceed through the subsequent steps leading to ATP production by the substrate level phosphorylation. This metabolic adaptation may account for both alanine and succinate accumulation under hypoxia and anoxia.

4.3. Nitrate, nitrite, and NO: Hemoglobin/nitric oxide cycle

In anoxic root cells, nonsymbiotic plant hemoglobins (Hbs) have been demonstrated to modulate the nitric oxide (NO) produced and the amount of

cytosolic nitrate, to be involved in a metabolic process regenerating NAD^+ , and to sustain ATP synthesis due to the reduction of nitrite to NO in mitochondria (Igamberdiev and Hill, 2009; Igamberdiev et al., 2005; Stoimenova et al., 2007).

In addition to the oxygen limitation, the enhanced NO production is also responsible for the inhibition of cytochrome oxidase at the oxygen-binding site (Cooper, 2002; Dordas et al., 2003). Briefly, due to the quantities of NO and oxygen, cytochrome *c* oxidase (COX) is not operative in donating electrons to oxygen, and nitrite may act as an alternative electron acceptor at sites of complexes III and IV of the electron transport chain (Stoimenova et al., 2007). Both of these complexes are proton pumping, and NO formation by means of nitrite reduction seems to be responsible for the proton translocation. Since ATP production is a consequence of this proton pumping, as shown in Fig. 5.4, plant mitochondria are

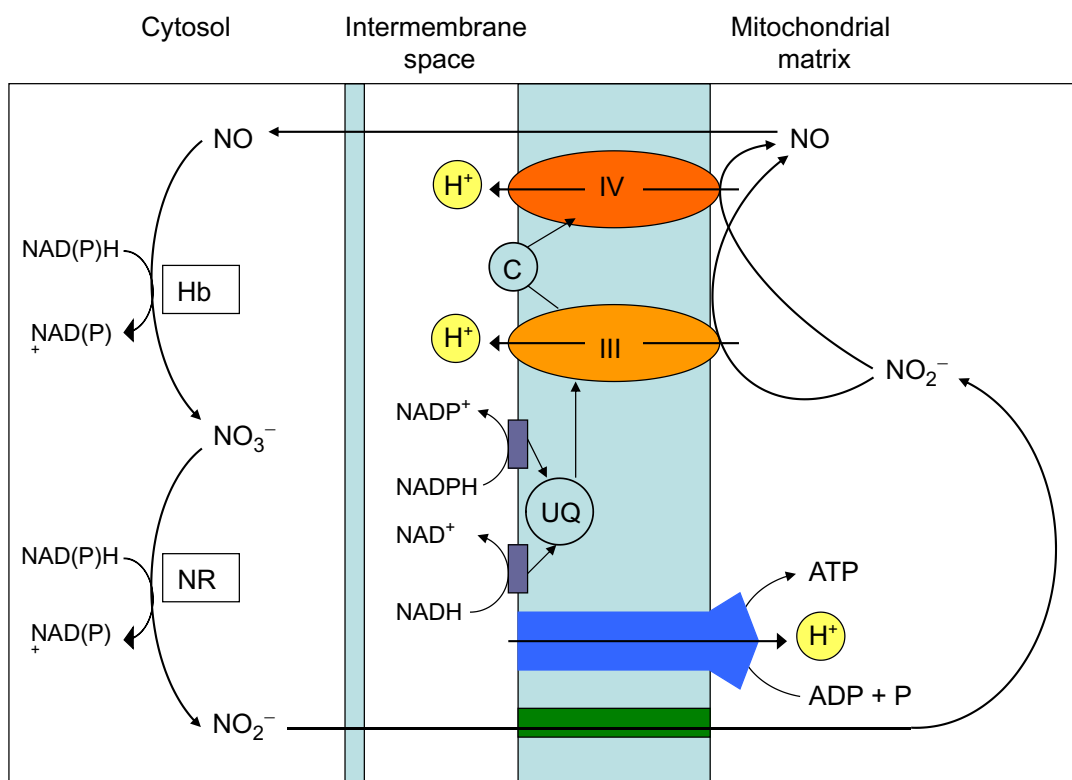


Figure 5.4 Anaerobic switch in plant mitochondria. Nitrite (NO_2^-) is the alternative electron acceptor at sites of complexes III and IV. The produced nitric oxide (NO) is converted to nitrate (NO_3^-) in the cytosol by means of hypoxically induced hemoglobin (Hb). Nitrate is then reduced to nitrite by nitrate reductase (NR) and then enters back the mitochondria. The cycle demonstrated to support the oxidation of NADH and NADPH and preserve the ATP production. C, cytochrome *c*; Hb, hemoglobin; NR, nitrate reductase; Q, ubiquinone.

able to synthesize anaerobic ATP by means of NADH and NADPH as electron donors and with nitrite as a terminal electron acceptor. In this mechanism, cytosolic Hbs play a fundamental role reacting with NO formed in the mitochondria and diffused to the cytosol-generating nitrate (Igamberdiev and Hill, 2009). In the cytoplasm, nitrate can then be reduced to nitrite by nitrate reductase (NR) and nitrite can enter mitochondria, allowing the cycle to repeat.

Effectively, in hypoxic-tolerant rice subjected to oxygen-deficiency stress, NR has been shown to be upregulated at both transcriptional and enzymatic levels, while nitrite reductase (NiR) shows a very low induction (Mattana et al., 1994). Moreover, the mitochondrial COX-dependent reducing systems seem to be highly active, suggesting that most of the nitrite enters mitochondria (Planchet and Kaiser, 2006). Only a very small percentage of the nitrite generated by means of the Hb/NO cycle seems to be reduced to NH_4^+ , even though ammonia is known to be fundamental for enhanced amino acid production under anaerobic conditions (Igamberdiev and Hill, 2009).

In particular, in roots of highly tolerant species, it has been shown how the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle operates in order to reassimilate and incorporate ammonia into those metabolites typical of anaerobic conditions (Reggiani et al., 2000), and GS, Fd-GOGAT, and Fd-NADP⁺ were demonstrated to be newly synthesized during anoxic stress (Mattana et al., 1994, 1996, 1997).

4.4. Lactate and ethanol: Anaerobic fermentation

Under anaerobic conditions, since the oxidative phosphorylation pathway is inhibited, alternative pathways are necessary to regenerate NAD⁺ in order to sustain glycolysis. Fermentation is one of the first metabolic adaptations which has been studied extensively, demonstrating that two fermentative pathways, ethanolic and lactic fermentations, can regenerate NAD⁺ from NADH.

It has been suggested that cytosolic pH is the trigger which regulates the ratio between ethanolic fermentation and lactic fermentation: a decrease in cytosolic pH increases ethanolic fermentation and decreases lactic fermentation (Davies et al., 1974; Menegus et al., 1991). Also Perata and Alpi, in 1993, sustained the hypothesis that lactate biosynthesis prevails in the early phases of anaerobic metabolism, causing a cytoplasmic acidification which stimulates the activity of pyruvate decarboxylase (PDC) and alcohol

dehydrogenase (ADH) with the subsequent ethanol formation. It has also been specified that there is not—as is true for most of the adaptive metabolic changes—a common rule for all the plants; some species do not show lactic acid production before ethanol production and ethanolic fermentation are the only fermentation pathway (Andreev and Vartapetian, 1992).

More recently, in 2008, Dolferus and colleagues reported that *Arabidopsis* plants that overexpress lactate dehydrogenase (LDH) demonstrated increased root tolerance to anaerobiosis (Dolferus et al., 2008). Interestingly, these mutants showed a higher activity of PDC, confirming that in *Arabidopsis* roots, the ethanolic fermentation pathway also depends on lactate production (Dolferus et al., 2008). In *Arabidopsis* plants, four genes encoding PDC have been found, and Kürsteiner and colleagues, in 2003, demonstrated that PDC1 and PDC2 mRNA levels are induced during oxygen deprivation (Kürsteiner et al., 2003), while the quantity of PDC3 and PDC4 genes is not influenced significantly when the plant is under hypoxic stress (Mithran et al., 2014). Also in 2014, Mithran and colleagues reported that these different isoforms are organ-specific—PDC1 is root specific, while PDC2 seems to play its role in leaves.

Furthermore, it has been reported that an overexpression of PDC1 and PDC2 results in an enhanced tolerance to oxygen deficiency (Ismond et al., 2003), while the *pdc1*-null mutants showed to worsen survival under the stress (Kürsteiner et al., 2003).

Concerning ADH, studies on both tolerant and intolerant species demonstrated that null or reduced ADH activity induces a higher sensitivity to the anaerobic condition. ADH knockout plants, *Arabidopsis adh1*-null mutants, rice with reduced ADH activity, and maize *adh1* mutants were in all of these cases more susceptible to anoxia (Ellis et al., 1999; Ismond et al., 2003; Jacobs et al., 1988; Johnson et al., 1994; Matsumura et al., 1995, 1998).



5. CONCLUDING REMARKS

Recent advances in plant strategies to survive anaerobic conditions gain knowledge about specific plant adaptive responses, increasing the understanding of how sensing, signaling, and thus response can quickly and successfully coordinate. The integrate regulation of molecular, metabolic, and physiological responses allows the plant survival even under strict low oxygen conditions.

Observation and deep analysis have highlighted different levels of the survival strategy, such as transcriptional and translational (i.e., TFs, such as

ERF VII and HSFs), posttranscriptional (i.e., polyribosomes), and posttranslational (i.e., NERP) regulation of specific genes involved in oxygen sensing and stress response triggering; accumulation of specific amino acids, such as alanine and γ -aminobutyric acid (GABA), succinate, and gamma-hydroxybutyrate (GHB), belonging to a complex reassessment of metabolic pathways with a role in the maintenance of the osmotic potential and limiting cytosolic acidification; and a temporary role for nitrite, where it acts as an alternative mitochondrial electron acceptor in the electron transport chain for the maintenance of the mitochondrial activity and ATP synthesis during anoxic conditions.

All of these new topics become pieces of a complex puzzle, charting new courses to deeply explore and understand plant survival mechanisms under anaerobic stress.

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