CHAPTER 1

Integrating classical and alternative respiratory pathways

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Introduction

Respiratory pathways are vital for plant carbon and energy metabolism, which is the main use of most assimilated carbohydrates. Most respiratory pathways are very well established, the prominent being glycolysis in cytosol and the tricarboxylic acid (TCA) cycle, which occurs in the matrix of mitochondria coupled with the electron transport chain (ETC) which functions along the inner mitochondrial membrane. Some glycolytic enzymes also associate with the mitochondrial membrane and dynamically support substrate channelling (Giegé et al., 2003; Graham et al., 2007). Despite cross-kingdom commonalities in glycolysis and the TCA cycle, the regulation of respiration is relatively poorly understood (Fernie et al., 2004) which reflects the complexity of respiratory pathways. In plants this complexity encompasses the only possibility of switching from glycolysis to fermentative metabolism but the utilization of alternative pathways in plants allows the maintenance of substrate oxidation while minimizing the production of ATP. Equally, new insights have suggested how ATP generation can be maintained under hypoxia. With this overview, this chapter will integrate such alternative respiratory pathways with components of the classical oxidative-phosphorylative pathways.
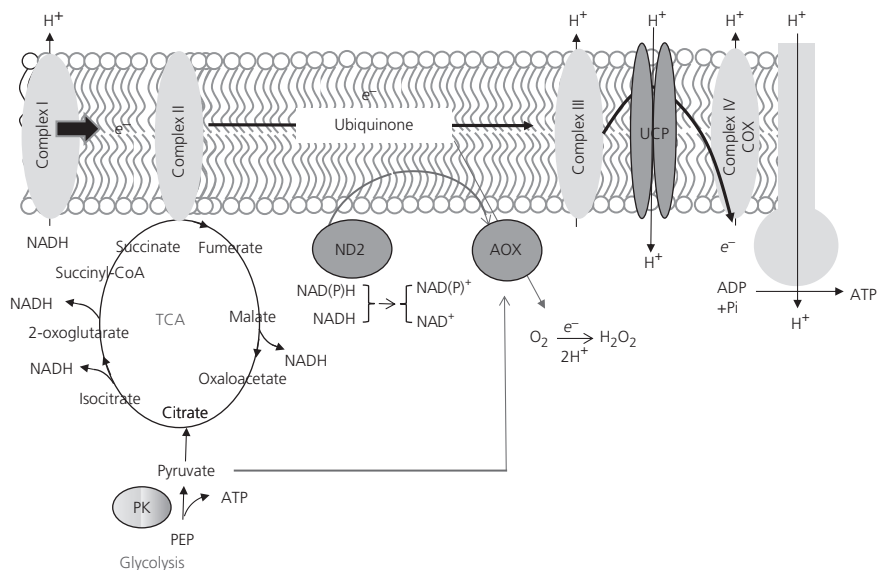
Mitochondrial electron transport generates ATP by using the reducing equivalents derived through the operation of the TCA-cycle. The classic operation of the ETC pathway involves the transport of electrons from such as NAD(P)H or succinate to oxygen via four integral membrane oxidoreductase complexes: NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome c reductase (complex III), cytochrome c oxidase (complex IV or COX), linked to a mobile electron transfer protein (cytochrome c) and ATP synthase complex (complex V). In complex V, the active extrusion of protons from the inner membrane space to the matrix leads to the generation of ATP (Boekema

Figure 1.1 Overview of electron transport chain dissipatory mechanisms in plant mitochondria.

and Braun, 2007) (Figure 1.1). Apart from this classical operation of the ETC, mitochondrial complexes interact to form so-called super-complexes or respirosomes (Boekema and Braun, 2007). Complex I, II and IV are involved in the formation of super-complexes with different degrees and configurations. It may be that the formation of super-complexes represents a regulatory mechanism that controls the passage of electrons through the ETC (Eubel *et al.*, 2003). Super-complex formation helps in increasing the stability of individual complexes, in the dense packing of complexes in the membrane and in fine tuning energy metabolism and ATP synthesis (Ramírez-Aguilar *et al.*, 2011).

Currently most research on alternative electron transfer is focused on non-phosphorylating bypass mechanisms: a second oxidase – the alternative oxidase (AOX), an external NAD(P)H dehydrogenases in the first part of ETC, and also plant uncoupling mitochondrial proteins (PUCPs).

Alternative oxidase (AOX)

AOX is located in the inner mitochondrial membrane of all plants and fungi and a limited number of protists. AOX also appears to be present in several pro-karyotes and even some animal systems (Chaudhuri and Hill, 1996; McDonald, 2008; McDonald and Vanlerberghe, 2006). Two forms of AOX are present in dicot plants (AOX1 and AOX2) while in monocots there is only one AOX (AOX1) (Considine *et al.*, 2002; Karpova *et al.*, 2002).

AOX are homodimeric proteins orientated towards the inner mitochondrial matrix. AOX diverts electrons from the main respiratory chain at the ubiquinone pool and mediates the four-electron reduction of oxygen to water (Figure 1.1). In comparison to electron transfer by the cytochrome chain (complex III and IV), AOX does not pump H^+, therefore transfer of electrons by AOX does not create a transmembrane potential, and the decline in free energy between ubiquinol and oxygen is dissipated and mostly released as heat (Vanlerberghe *et al.*, 1999). The diversion of electrons to the AOX pathway can reduce ATP generation by up to 60% (Rasmussen *et al.*, 2008). The AOX ATP dissipatory pathway plays an important role when the ETC is inhibited by various stress conditions. ETC inhibition increases $NADH/NAD^+$ and ATP/ADP ratios and as a consequence the TCA cycle could slow down. In addition to the energetic consequences of this, the number of carbon skeletons being produced will also be limited as the export of citrate supports nitrogen assimilation. Against this, AOX contributes to the maintenance of electron flow and the production of reducing equivalents to help maintain the TCA cycle. Indeed, AOX activation occurs in direct response to stress. A feature of all stress conditions is an increase in the production of reactive oxygen species (ROS): a process that can occur from the over-reduction of cytochrome components through the disruption of the ETC. In response to this, ROS or ROS-induced signals such as salicylic acid, act to induce the transcription of AOX (Vanlerberghe and McIntosh, 1997; Mackenzie and McIntosh, 1999) as also suggested from the observation that the addition of antioxidants leads to the suppression of AOX (Maxwell *et al.*, 2002).

Oxygen, AOX and COX

Once induced by ROS, AOX may function as a negative feedback mechanism to suppress ROS production; a feature that we have named oxygen homeostasis (Gupta *et al.*, 2009). This feedback mechanism is a consequence of large differences in O_2 affinities of the classical and alternative respiratory pathways. The K_m of COX is approximately 0.1 µmol but in AOX it is between 10 and 20 µmol (although the study by Millar *et al.*, 1993 suggested a 10-fold higher AOX affinity for O_2). Given these affinities, COX will maintain respiration whilst AOX reduces the O_2 concentration, thereby decreasing the production of ROS inside the mitochondrion (Puntarulo and Cederbaum, 1988; Skutnik and Rychter, 2009). This is supported by the observations of Ribas-Carbo *et al.* (1995) who used an oxygen isotope discrimination technique to show that the inhibition of AOX by its inhibitor salicylhydroxamic acid (SHAM) did not lead to a decrease in total respiratory rates. This mechanism would be an exception to the 'energy over flow' model proposed by Lambers (1982), who suggested that in certain situations (e.g. excess carbohydrate), non-phosphorylating alternative pathways might contribute significantly to total respiration. Oxygen homeostasis could be of especial relevance in situations where different plant

tissues are subjected to fluctuating O_2 concentration due to diffusion gradients, and more so under environmental conditions such as flooding (Rolletschek *et al.*, 2002; Bailey-Serres and Chang, 2005; Schmälzlin *et al.*, 2005; Bailey-Serres and Voesenek, 2008; Rasmusson *et al.*, 2008).

The electron partitioning model of Ribas-Carbo *et al.* (1995) suggests that COX and AOX compete for electron and electron passage but this must be influenced by the stress response of each pathway and particularly if exposed to low partial pressures of O_2 (Po_2). In a study undertaken by the senior author's group, root slices of several species were incubated in a sealed cuvette and the respiratory rate of the tissue was measured until total oxygen was depleted in the vial. Until a partial pressure of 4% Po_2, the decrease in respiratory rate correlated linearly with O_2 concentration; however, at <4% Po_2 level, the respiratory oxygen consumption rate slowed, taking a longer time to consume oxygen, indicating that a more slowly respiring plant would promote survival under the latter condition (Zabalza *et al.*, 2009). This unique phenomenon has been named as the 'adaptive response of plant respiration (ARPR) to hypoxia'. To determine which among the respiratory pathways could be influencing ARPR, each pathway was selectively inhibited in hydroponically grown pea using either KCN (an inhibitor for COX) or SHAM (an inhibitor for AOX). When AOX was the only electron acceptor, O_2 consumption continued without any alteration until all the oxygen was depleted, but when AOX was inhibited, ARPR was still observed. Thus, the COX pathway was found to be responsible for ARPR (Zabalza *et al.*, 2009). Clearly, ARPR is not a consequence of differentially responsive O_2 affinities of the terminal oxidases (see earlier) as it occurs at Po_2 above the K_m of both oxidases. The decline in respiration could not be explained by a depletion of carbohydrates, as respiratory substrates, since when the same root material was immediately reused in experiments, ARPR was still observed. Moreover, oxygen diffusion through the tissue was not limiting at low Po_2 because ARPR was also observed with in single-celled organism *Chlamydomonas* which has a diameter approximately 20 μm (Gupta *et al.*, 2009). The lower Po_2 was not in itself limiting respiratory rates as respiration could be elevated by the prior addition of 10 mM pyruvate prior to assessing ARPR. Taken together, these observations point towards the most likely scenario of the existence of an oxygen sensing mechanism that regulates the rate of mitochondrial oxygen consumption at low Po_2.

Pyruvate kinases, classical respiratory metabolism and AOX

Pyruvate kinase (PK; EC 2.7.1.40) plays a critical role in glycolytic pathway catalyzing the terminal reaction of the glycolytic pathway by converting ADP and phosphoenolpyruvate (PEP) to ATP and pyruvate. As pyruvate regulates both glycolysis and the TCA cycle (Pilkis and Granner, 1992; Teusink *et al.*, 2000), PK represents a crucial respiratory regulatory node. PK exists as tissue-specific isozymes that exhibit significant differences in their physical and kinetic properties

(reviewed by Plaxton and Podesta, 2006). This reflects the presence of different PK isozymes in the cytosolic and plastidial compartments in plants; designated as PKc and PKp forms respectively (Plaxton, 1996; Givan, 1999). Transgenic tobacco plants which were deficient in PKc were used to demonstrate its role in regulating development via modulation of carbon sink-source relationships (Knowles *et al.*, 1998; Grodzinski *et al.*,1999). PKc lines exhibited delayed shoot and flower development and this was correlated with poor export of previously fixed $^{14}CO_2$ from leaves in the 'night-time' phase of a light-dark cycle but increased $^{14}CO_2$ release from respiration (Grodzinski *et al.*,1999). Conversely, in another study with *Arabidopsis* seeds, PKp has been shown to play an important role in fatty acid biosynthesis (Andre and Benning, 2007; Andre *et al.*, 2007). PKs also exist as tissue specific isozymes (Turner *et al.*, 2005). The subtle respiratory regulation that these difference in PK isoforms affords is well-illustrated by a classic study of PKc repression and activation in castor seed endosperm (Podesta and Plaxton, 1991). In castor seeds, during aerobic conditions, the allosteric inhibition of endosperm PKc facilitated larger gluconeogenic conversion of stored triacylglycerides to hexose-phosphates assisting in germination. However, under low oxygen PKc became active in order to compensate for ATP depletion that occurs due to hypoxic stress (Podesta and Plaxton, 1991).

A key study also used a transgenic approach to provide greater insight into the role of PKc in carbon metabolism through the coordinated regulation of glycolysis, the TCA cycle, the mitochondrial ETC and also AOX in potato tuber (Oliver *et al.*, 2008). A role for PKc in these respiratory pathways was implied from a series of observations. Firstly, pyruvate addition experiments showed an effect on glycolytic flux and the consequences that altered the dynamics of mitochondrial ETC (Zabalza *et al.*, 2009). The link to AOX was suggested when an increase in AOX activity was seen after pyruvate was added to isolated mitochondria (Millar *et al.*, 2003). This AOX effect was then explained through the interaction of pyruvate to cysteine residue of AOX (Umbach *et al.*, 2006).

Transgenic potato tubers with decreased in PKc levels were generated through an RNA interference (RNAi) gene silencing approach, among which three lines were selected, lines PKC-25, 6 and 15 – where PK activity was reduced to ~40%, 37% and 29% respectively (Oliver *et al.*, 2008). As expected, lowering PKc expression led to a higher PEP to pyruvate ratio in actively growing tubers. This decrease in pyruvate levels correlated with a decrease in the various organic acids involved in the TCA cycle and there was also a decrease in the level of total protein in the tubers. [^{14}C]Glc labelling and feeding experiments showed a slight decrease in carbon partitioning towards organic acid and protein synthesis upon decrease in PKc levels. These results clearly demonstrated that PKc plays a very important role in the regulation of the levels of organic acids in tubers and partitioning the carbon toward the TCA cycle but interestingly total respiration and TCA cycle flux did not alter. One reason could be that residual pyruvate levels are probably enough to maintain the respiratory activity in these tubers. Equally,

other enzymes that generate pyruvate such as PKp, PEPC, or PEP phosphatase could be compensating for the loss in PKc. Alternatively; there could be a compensatory change in electron transport through the COX pathway, which is in line with the electron partition model (Ribas-Carbo et al., 1995). This would imply that respiratory metabolism has a high homeostatic ability allowing considerable flexibility in response to changes in metabolite and transcript levels (Nunes-Nesi et al., 2005, 2007; Studart-Guimarães et al., 2007).

The potato RNAi lines also exhibited a suppression of AOX-dependent respiration which could be reversed by external feeding of pyruvate to tuber tissue. Suppression of the AOX pathway would be beneficial in growing tubers, which characteristically have low internal oxygen concentrations and low adenylate energy charge (Geigenberger, 2003). In line with this, PKc silenced plants produced significantly more tubers which also tended to be larger than the control tubers (Oliver et al., 2008). Thus, PKc modulation of pyruvate accumulation would be of great agronomic importance, functioning as a key regulatory step in potato tuber development by influencing the AOX in heterotrophic potato tubers.

NADPH dehydogenases linked to AOX

In addition to complex I (NADH dehydrogenase) there are some additional proteins which can use NADH and NADPH to reduce ubiquinone pool. There are NAD(P)H dehydrogenases. Type II NAD(P)H dehydrogenases (ND2) are membrane-bound proteins that face either the matrix or the inter-membrane side (Figure 1.1). Unlike complex I these are not involved in proton translocation and therefore do not contribute for ATP synthesis. As shown in Figure 1.1 there are at least four types of NADH dehydrogenase proteins; two on the external side of the inner mitochondrial membrane (one oxidizing NADH and one NADPH) and two to the inner face of the inner membrane (similarly one devoted use NADH and other use NADPH) (Rasmusson and Møller, 1991). Substrate specificity for these dehydrogenases is based on pH and calcium. Since various environmental conditions and biotic abiotic stresses influence the dynamics of calcium and pH, which in turn have cascading effects on activities of NADH and NADPH dehydrogenases (Felle, 2005; Dodd et al., 2010). For instance NADPH dehydrogenases are involved in nitric oxide generation under anoxia. In view of these intricate dynamic processes, uncovering the roles of different dehydrogenases has been an area of intense research (Michalecka et al., 2003; Rasmusson et al., 2008). There are reports that specificity for NADPH of the external NADPH dehydrogenase NDB1 at low pH becomes important under hypoxia (Felle, 2005). This leads to oxidation of cytosolic NADH under hypoxia which leads to recycling of NAD^+.

Uncoupling proteins (UCPs)

Plant uncoupling proteins are a class of mitochondrial anion carrier proteins. UCP is a specialized protein that uncouples electron transport from ATP synthesis in mitochondria by acting downstream of complex IV (Figure 1.1). The primary functions of UCPs are to transport protons from the intermembrane space into the mitochondrial matrix. This translocation leads to generation of electrochemical gradient ($\Delta\psi$) (Rial *et al.*, 1983) which is opposite of ATP and this action leads to a decrease in $\Delta\psi$, and the potential energy of the $\Delta\psi$ is dissipated as heat (Vercesi *et al.*, 2006). Therefore UCPs were initially considered as energy wastage proteins. UCP mediates a fatty acid dependent, purine nucleotide-inhibited proton leakage across the inner mitochondrial membrane (Krauss *et al.*, 2005). Therefore, within the context of plant energy-balance rearrangements, UCP may have overlapping functions with other alternative pathway proteins in the ETC like AOX and NAD(P)H dehydrogenases. Due to this, a tight regulation of UCP takes place in mitochondria. UCPs are mainly activated by free fatty acids and activity diminishes by ADP, GDP, ATP and GTP; (Vercesi *et al.*, 1995; Jezek *et al.*, 1996). Various physiological states such as pH, redox status of the ubiquinone pool control UCPs activity (Navet *et al.*, 2005; Borecký *et al.*, 2001). For instance, a decline in pH from 7.1 to 6.3 promotes the inhibitory effect of UCPs (Borecký *et al.*, 2001). It was also found that ROS can increase the activity of UCP. First interaction of ROS with membrane lipids leads to the production of 4-hydroxy-2-trans-nonenal which then activates the proton translocation activity of the UCPs (Smith *et al.*, 2004). UCPs are known to protect plants from high light, drought or heat stress. Supporting evidence in line with this is that the over-expression of Arabidopsis UCP (AtUCP1) in tobacco suppressed drought and salt stress-associated respiration. The AtUCP1 transgenic lines exhibited lower levels of ROS and higher tolerance to drought and salt stress (Begcy *et al.*, 2011). Not only to combat stress, UCPs also facilitate the synthesis of intermediates for amino acid and lipid biosynthesis (Tielens and Van Hellemond, 1998; Sweetlove *et al.*, 2007). This is via increasing metabolic flux during the conditions of excess ATP production by, for example, photosynthetic light reactions. Sweetlove *et al.* (2007) demonstrated that UCPs are involved in the recycling of metabolic intermediates of photorespiration and play important role in maintaining the metabolite flux during the condition of photorespiration.

## Electron transfer flavoprotein (ETF)

Besides uncovering pathways which remove excess reducing power and balance the redox poise of the cell, several additional electron donors to the mitochondrial ETC in addition to NADH and NADPH have been uncovered in plants.

Most of them are similarities to well-characterized animal systems (Fe, 1988; Frerman *et al.*, 2001). One of such components is the electron transfer flavoprotein (ETF). ETF was first identified by Crane and Beinert in 1956 based on its capability to transfer electrons to various acceptors from fatty acyl-CoA dehydrogenases. Mammalian ETF is a heterodimer of alpha and beta subunits which are 31 and 27 kDa respectively, each binding to a single flavin adenine dinucleotide (FAD) as a redox responsive co-factor (McKean, Beckmann and Frerman, 1983). This protein is located in mitochondrial matrix and encoded by nuclear genome. ETF is an electron acceptor for at least nine mitochondrial matrix flavoprotein dehydrogenases. These are four straight fatty acyl-CoA dehydrogenases and five dehydrogenases which are involved in the catabolism of amino acids such as glutaryl, isovaleryl short and long chain and choline (reviewed by Roberts *et al.*, 1996 and the literature therein). These donors can be also classified as seven acyl-CoA dehydrogenases and two N-methyl dehydrogenases, isovaleryl-CoA dehydrogenase (IVDH), 2-methyl branched-chain acyl-CoA dehydrogenase, glutaryl-CoA dehydrogenase, sarcosine and dimethylglycine dehydrogenases. ETF donates electrons to flavoprotein:ubiquinone oxidoreductase (ETFQO) which are transferred to the ubiquinone pool (Ishizaki *et al.*, 2005). In mammalian systems the ETF-EFFQO has been shown to link the β-oxidation of fatty acids, choline and various amino acids to respiratory metabolism (Frerman, 1987). As a result mutation in either ETF or ETFQO leads to type II glutaric acidemia disease in humans where the build-up of incomplete processed proteins and fats leads to blood plasma acidosis (Frerman and Goodman, 2001).

Within plant science ETF came into picture when Heazlewood *et al.* (2004) identified the ETF system by liquid chromatography, tandem mass spectrometry mitochondrion proteomic analysis of *Arabidopsis*. Very soon afterwards *Arabidopsis* genes encoding ETFQO were discovered (Ishizaki *et al.*, 2005). It quickly emerged that the ETF-ETFQO system was involved in plant senescence which includes lipid mobilisation. Thus, Buchanan-Wollaston *et al.*, (2005) found the ETF system was transcriptionally induced during dark-induced senescence but this role was unambiguously demonstrated with T-DNA tagged mutants in *Arabidopsis* (Ishizaki *et al.*, 2005, 2006). Both ETF and ETFQO T-DNA mutants exhibited accelerated senescence and early death compared to wild-type during extended darkness. Interestingly, the mutants exhibited altered amino acid metabolism and in particular the accumulation of a leucine catabolism intermediate (Ishizaki *et al.*, 2005, 2006). The ETC complex was induced by oxidative stress following menadione treatment (Lehmann *et al.*, 2009) and it is tempting to suggest that senescence-associated oxidative stress triggers the ETC to contribute towards the energetic demands of cellular catabolism. Indeed, phytol and branched chain amino acid degradation leads to the formation of isovaleryl-CoA which can be oxidized by isovaleryl dehydrogenase (IVDH)
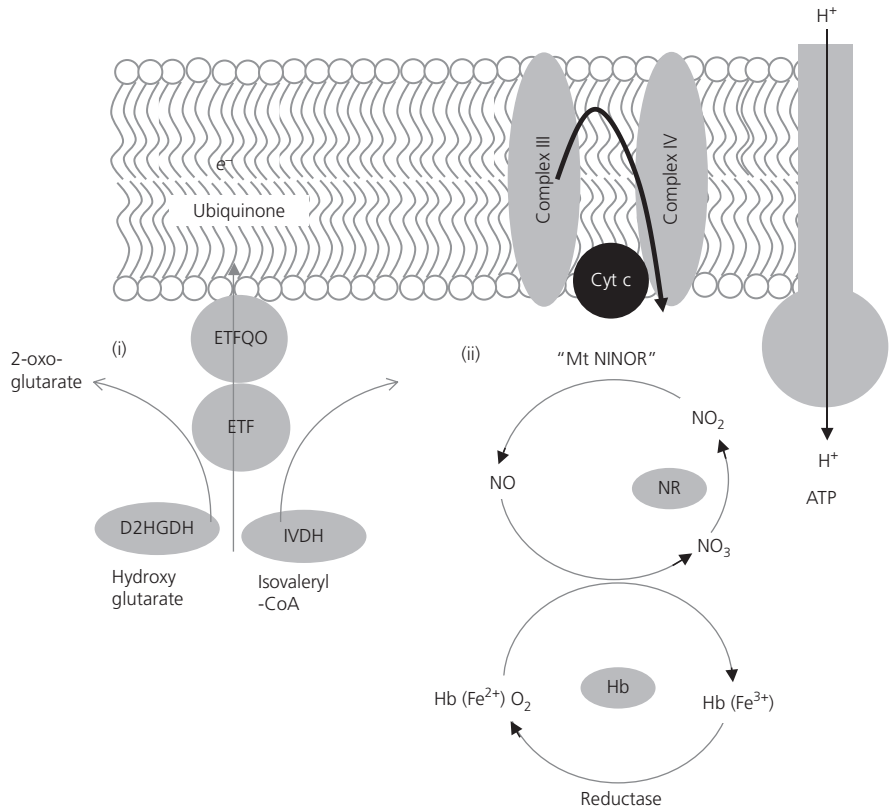
Figure 1.2 Alternative ATP generating mechanisms via operation of ETF/ETFQO and hemoglobin nitric oxide cycle.

leading to a transfer of electrons to the ETF/ETFQO system (Araújo *et al.*, 2010). Similarly, hydroxyglutarate formed via lysine degradation is oxidized by 2-hydroxyglutarate dehydrogenase (D2HGDH) to 2-oxo-glutarate (2-OG) to transfer electrons to ETF (Figure 1.2). The relative importance of each pathway in ETF/ETFQO expression has been investigated using knockout mutants of IVDH and D2HGDH, both enzymes being encoded by single genes (Araújo *et al.*, 2010). Comparing continuous light (24 h light), short day (8 h light/16 h dark) and in cold conditions (13 °C, 16 h light/8 h dark) *ivdh-1* plants exhibited a clearer accelerated senescence than the *d2hgdh1–2* plants. This finding suggests that IVDH is more likely to control the provision of electrons to the ETF/ETFQO complex than D2HGDH. Lysine was found to accumulate in both mutants, implying that this amino acid accumulation is important to flux the electrons through the ETF/ETFQO complex.

Deploying electron dissipatory mechanisms whilst maintaining ATP production under stress situations

Stress imposes certain conditions in plants during which ETC components can become over-reduced to produce ROS and electron dissipation becomes vital but ATP production is still required for energy requirement. Due to the situation of O_2 limitation, hypoxia represents a fascinating interplay between aerobic and anaerobic respiratory metabolism that is discussed here.

Hypoxia is one of the barriers for respiration in bulky tissues (Rolletschek *et al.*, 2003; Rolletschek *et al.*, 2005a, 2005b, 2007) but also plants experience hypoxia that lead to alterations in respiration, for instance during the period of flooding or waterlogging. O_2 depletion occurs where respiration dominates over O_2 availability that result in the depletion of ATP (Zabalza *et al.*, 2009; van Dongen *et al.*, 2009). One major structural change that occurs in certain plant roots is the formation of aerenchyma (Drew *et al.*, 2000). However, this is mediated by ethylene whose biosynthesis is dependent on O_2-requiring ACC oxidase so aerenchyma tend to form only under hypoxic conditions (He *et al.*, 1996). In anoxic conditions aerenchyma formation might takes place only with active photosynthesis which can transfer O_2 to the roots. The metabolic adjustment to low oxygen includes the down-regulation of energy–consuming metabolic pathways (Geigenberger, 2003; van Dongen *et al.*, 2011 that include the down-regulation of storage carbohydrate metabolism (Geigenberger *et al.*, 2000), the metabolic shift from invertase to sucrose synthase pathway (Bologa *et al.*, 2003; Huang *et al.*, 2008), and the inhibition of mitochondrial respiration at near low oxygen to utilize available oxygen for longer time (Gupta *et al.*, 2009; Zabalza *et al.*, 2009). Downregulation of energy inefficient pathways such as AOX pathway also takes place at low oxygen which is a part of the plant survival strategy. When the O_2 concentration decreases below the level of operation of oxidative phosphorylation, plant cells follow various alternative strategies to produce ATP. These include the operation of glycolytic pathway (even in low oxygen situations), which produces two ATP and two pyruvate molecules per unit of hexose utilizing while concomitantly reducing NAD^+ to NADH. However, for the glycolytic pathway to operate NAD^+ must be continuously regenerated from NADH via fermentative pathways. By using pyruvate as substrate, fermentative metabolism either produces ethanol via pyruvate decarboxylase (*Pdc*) and alcohol dehydrogenase (*Adh*) or lactate via lactate dehydrogenase (Tadege *et al.*, 1999). It seems likely that these pathways play role in hypoxic survival as both that *Pdc* and *Adh* are strongly induced in response to this stress (Rahman *et al.*, 2001; Kürsteiner *et al.*, 2003). However lactate and ethanol are potentially cytotoxic, if produced in high concentrations (Figure 1.3).
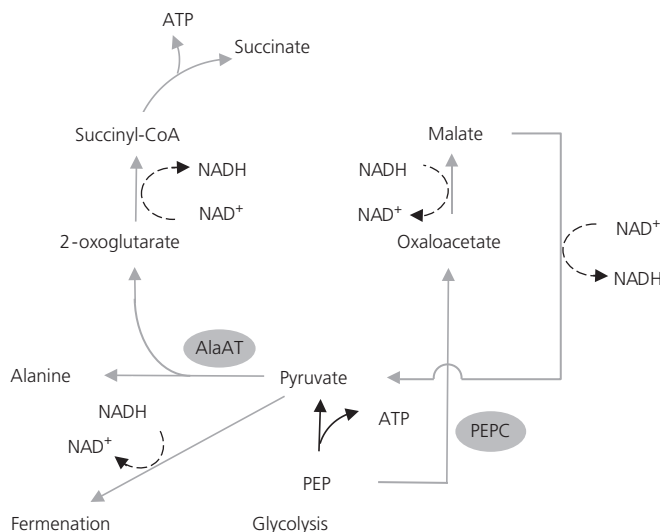
Figure 1.3 Reconfigured TCA metabolism during hypoxia via alanine aminotranferase.

Another important chemical induced at low oxygen is nitric oxide (Planchet *et al.*, 2005). NO production by mitochondria leads to NAD(P)H consumption and the generation of a limited amount of ATP under anoxic conditions (Stiomenova *et al.*, 2007), via operation of haemoglobin (Hb)-NO cycle (Figure 1.2). Non-symbiotic Hbs ($NO+O_2 \rightarrow NO_3^-$) have a high affinity for oxygen; over two orders of magnitude lower than that of COX which allows a limited respiration at very low Po_2. NO oxidation by Hb results in the formation of oxidized ferric metHb [$Hb(Fe^{3+})$] and so the reaction is ($Hb(Fe^{2+})O_2+NO+\rightarrow Hb(Fe^{3+})+NO_3^-$). The Hb is then reduced to its ferrous form [$Hb(Fe^{2+})$] by an associated reductase. NO_3^- is reduced to NO_2^- by nitrate reductase ($NO_3^-+NAD(P)H\rightarrow NO_2^-+NAD(P)^+ + OH^-$) and NO_2^- is reduced back to NO by mitochondrial nitrite NO-reductase activity (Mt NINOR) at complex III and cytochrome *c* oxidase ($2NO_2^-+H^+ + NAD(P)H\rightarrow 2NO+NAD(P)^+ + 2OH^-$) donates electrons to the ETC and also restarts the cycle (Igamberdiev and Hill, 2009). Crucially, the Hb-Mt-NINOR cycle only comes into play when the O_2 concentration falls below $2\,\mu M$ and so appears to be particularly tailored to confer tolerance during anoxic conditions (Gupta *et al.*, 2005).

Alanine is a metabolite that accumulates at high concentrations at low Po_2 (de Sousa and Sodek, 2003) and under hypoxia, alanine comprised 50% of the soluble amino acid fraction of excised rice roots representing 1.2% of the root dry weight (Reggiani *et al.*, 1988). Recent ^{15}N labelling experiments suggested that while N uptake was reduced, amino acid metabolism was redirected towards alanine and γ-aminobutyric acid synthesis (Oliveira and Sodek, 2013). This substantial production of alanine is driven by alanine aminotransferase (AlaAT) (EC 2.6.1.2) which catalyses the reaction between pyruvate and glutamate to form

alanine and 2-oxoglutarate (2-OG). In *Arabidopsis* there are two sequences that code for the AlaAT; with AlaAT-1 likely targeted to the cytosol and AlaAT-2 to the mitochondria (Liepman and Olsen, 2003). Two subclasses of AlaAT have been extensively characterized in soybean plants that were exposed to hypoxic stress with different nitrogen sources. Semi-quantitative PCR expression analysis showed that AlaAT were highly expressed in hypoxic roots and nodules. Reoxygenation caused a decrease in transcript and alanine content without altering the activity of enzyme, possibly suggesting an allosteric control mechanism operating under such conditions. Under NH_4^+ nutrition, the transcript abundance and enzyme activities were found to be higher in comparison to NO_3^- nutrition (Rocha *et al.*, 2010a, 2010b). Further, by using AlaAT T-DNA knockout plants, it was demonstrated that alanine production does not purely depend on these enzymes (Miyashita *et al.*, 2007), and that alanine can also be made by γ-aminobutyric acid transaminase (GABA-T) using pyruvate as co-substrate (Miyashita and Good, 2008). Obviously, the next central question would be on the role of alanine in hypoxia. The active transport of the accumulated alanine to the shoot via the xylem after the flooding period suggests that the recycling of alanine takes place after flooding. This may improve carbon and nitrogen distribution after flooding, conferring faster recovery of the plant (de Sousa and Sodek, 2003). Drew (1997) suggested that the accumulated alanine could improve energy-producing efficiency via the glycolytic flux, thereby assisting plant survival during hypoxic conditions. However, this argument is defeated by the fact that AlaAT-mediated alanine production is not coupled to NAD(P)H to regenerate NAD^+, as is the case with such fermentative pathways. An alternative suggestion that alanine accumulation might serve to buffer the pH in anoxic cells was made by Reggiani (1988). However, the most obvious metabolic role for alanine accumulation is the prevention of excess pyruvate accumulation which could impact on AOX activity (Zabalza *et al.*, 2009). In the absence of AlaAT activity, a pyruvate-driven increase in respiration could deplete internal O_2, instead of the required decrease in O_2 consumption needed for short-term plant survival (Gupta *et al.*, 2009). Therefore, alanine accumulation serves as an indirect survival strategy evolved by plant cells as a response to hypoxic stress (Rocha *et al.*, 2010a, 2010b)

Conclusions

To conclude, this chapter provides an overview, illustrating the functional flexibility of classical and alternative respiratory metabolism that coordinate with high precision to maintain ATP generation under a range of situations that could otherwise lead to an over-reduction in ETC components, and more so during hypoxia. As such it is clear that understanding the pathways and their interactions during various environmental conditions is an essential

prerequisite to any appreciation of plant physiology and, thus, topics such as crop yield. The chapters in this book expand many of these themes, which are fundamental to plant biology.

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