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## Supplementary Material

### **Energy-crises in well-aerated and anoxic tissue: does tolerance require the same specific proteins and energy-efficient transport?**

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**Supplement 1** *Hydrophilic compounds reduce ATP levels*

This supplement concerns the idea of Frenkel and Eretz (1996) that ethanol would promote the fluidity of membranes and hence explain the higher tolerance of subsequent exposure to 2°C. However, several lipophilic anaesthetics are known to reduce ATP levels and by inference ATP production (Sztark *et al.* 1999): as one example the lipophilic anaesthetic bupivacaine reduced ATP and the total ADN by 40-50 % (Sztark *et al.* 1994). This reduction in oxidative phosphorylation may lead to acclimation and consequently higher tolerance to a subsequent treatment, which would require acclimation to an energy deficit.

**Supplement 2** *Some detailed comments on the question of energy provision by the battery, or by the PM-H<sup>+</sup> PPIase*

The main text on K<sup>+</sup> fluxes and sugar uptake, as related to the possible mechanism of transport, was mainly derived from different experiments and with different time intervals, so they can only be considered as indicative.

For the storage red beet root slices, the only sugar uptake measurements available were for the 2<sup>nd</sup> h after start of anoxia, when the sucrose uptake was 0.4 μmol g<sup>-1</sup> fresh weight h<sup>-1</sup> (Zhang and Greenway, 1995), with a K<sup>+</sup> loss during this period of 0.6 μmol g<sup>-1</sup> fresh weight h<sup>-1</sup> (Zhang *et al.* 1992). Thus, for that period the indication is that the battery was more than sufficient to cope with any energy required for the sucrose uptake even if the coupling ratio sugar/H<sup>+</sup> was an energy inefficient 1.0. In other words, during an energy crisis there was use of energy locked in K<sup>+</sup>- organic acid, produced during a period of high energy production. There are no measurements for sugar uptake over long term anoxia, but judged by persistent K<sup>+</sup> losses over 250 h anoxia, without any sign of injury, the battery might have continued to supply energy for substrate uptake over that period.

In contrast, excised rice coleoptiles between 17.00-17.30 h after start of anoxia, absorbed sucrose or glucose at a rate 0.65 μmol g<sup>-1</sup> fresh weight h<sup>-1</sup> (Exogenous sugar concentration of 0.5 mM). In the same experiments, there was over the full 17 h anoxia, net K<sup>+</sup> uptake of 0.1-0.14 μmol g<sup>-1</sup> fresh weight h<sup>-1</sup>. So, since there was net K<sup>+</sup> uptake rather than loss, no battery was involved during the sugar uptake, which presumably depended on energy produced by a low but sustained activity of the PM-ATPase.

These tentative data need further testing, at least by testing sugar uptake and concurrent fluxes of K<sup>+</sup>, over the full experimental period, probably best by using a sugar analogue.

**Supplement 3** *Comments on observed increase in tolerance in anoxia intolerant Arabidopsis due to absence of the 'O<sub>2</sub> sensitive' branch of N-end rule pathway*

The N-end rule pathway functions in selective protein degradation (Graciet and Welmer, 2010); enzymes to be degraded will have terminal amino acid residues oxidised, such as methionine and cysteine and then become destabilised, i.e. become substrates for the protein degrading N-end rule pathway. For example, during plant development, plant senescence and as suggested by Gibbs *et al.* (2011) during O<sub>2</sub> deprivation. This suggestion is reasonable in view of the substantial change in metabolism during hypoxia-anoxia (Felle 2005, Chang *et al.* 2000).

Two sets of data indicate the importance of the N-end rule pathway during O<sub>2</sub> deprivation under their particular conditions.

Firstly, *Arabidopsis* mutants which had no functional 'O<sub>2</sub> sensitive' branch N-end rule pathway, germinated much better than the wild type at 3 % O<sub>2</sub>, while also being less damaged after an anoxic shock followed by 6-9 h anoxia (Gibbs *et al.* 2011). In contrast, there was no difference between wild type and mutants when first acclimated by a hypoxic pre-treatment. The mutants without a functional O<sub>2</sub> sensitive branch of N-end rule pathway contained constitutive ADH, PDC and sucrose synthase, 3 critical enzymes of energy crisis metabolism (Gibbs *et al.* 2011).

In the second experiment *Arabidopsis* mutants of an ethylene responsive transcription factor (ERF), which are much less readily degraded by the N-end rule pathway, germinated much better than the wild type at 3% O<sub>2</sub>. Importantly one of these mutants also survived anoxia better. However, the mutants grew much less under aerated conditions (Paul *et al.* 2016).

Summing up: Following anoxic shock the mutants did perform better, but that was at expense of much reduced development under aeration. The increased tolerance to anoxic shock would not be relevant too most field situations, anoxia is nearly always preceded by a period of hypoxia (Drew, 1997).

The experiments are of substantial physiological-molecular biology value. However, the Gibbs experiments do not give a clue why anoxia intolerant tissues are distinct from anoxia tolerant tissues.

**Supplement 4** *Some relevant examples of defects in techniques which hinder interpretation*

Problems with techniques which hinder interpretation were discussed in detail by Gibbs and Greenway (2003). Some further examples relevant to the present review are added here, not only in the hope that future research will avoid such pitfalls, but also to avoid erroneous implications from data in the existing literature.

#### *4.1 Experiments with Aribidopsis,*

The experiments by Gibbs *et al.* (2011) are a good example of limitations rather common for experiments with this valuable species. In the Gibbs paper these inadequacies are: 1) even the most tolerant mutant scored 3 on a scale from 1-5, a scale based on appearance including greenness, after 12 h anoxia and 3 days air. As stated in our main text more definitive tests within minutes, or at most a few h, after return to air would be much more informative, such as membrane potential, elongation growth, or uptake of  $\text{Cl}^-$  from concentrations between 0.5 and 1 mM, a concentration at which it is certain  $\text{Cl}^-$  accumulation requires energy and intact tissues.

#### *4.2 Experiments with Nitrate reductase*

For a more detailed evaluation of  $\text{NO}_3^-$  reduction, it is important that methodology in experiments on  $\text{NO}_3^-$  reduction are often inadequate. For example the often quoted paper by Lee (1979) which showed that under anoxia 85 % of the  $\text{NO}_2^-$  formed from  $\text{NO}_3^-$  leaked to the medium mainly used excised roots, which were not healed and were anoxically shocked. Similar problems exist in the experiments by Brotel and Kaiser (1997). These cases demonstrate the predicament, without data obtained with better techniques the data by Lee and by Brotel and Kaiser(1997) remain in doubt, yet in the absence of data obtained with proper procedures we decided it was worth quoting them.

#### ***Supplement 5***

*Establishing a suitable exogenous  $\text{O}_2$  concentration to acclimate roots before exposure to anoxia.* This supplement was also published in the review by Atwell *et al.* (2015). It is reprinted here in view of the very different  $\text{O}_2$  concentrations which may be required for different tissues. The large improvement in anoxia tolerance induced during acclimating root tissues by exposure to hypoxia prior to anoxia (removing all  $\text{O}_2$ ) has been demonstrated (first by Saglio *et al.* 1988) and re-emphasized by Gibbs and Greenway (2003). The type of hypoxic pre-treatment should be established for each particular tissue or cell suspension, since internal  $\text{O}_2$  status depends on tissue thickness, turbulence, respiration rate and temperature (Berry and Norris 1949, Armstrong and Beckett, 1987; Gibbs and Greenway, 2003). In order to establish the optimum exogenous  $\text{O}_2$  concentration for acclimation, an  $\text{O}_2$  response curve ( $\text{O}_2$  uptake vs external  $\text{O}_2$  concentration) in the form of a hyperbolic curve should be used to choose a concentration that achieves half-maximal saturation (i.e. close to the  $K_m$ ).

## Supplement 6.

### Definitions

Abbreviation/term	Definition
AEC: energy charge	$(ATP + 0.5 ADP)/(ATP + ADP + AMP)$ .
Anoxic core	When during hypoxia certain tissues become 'anoxic' (i.e. anaerobic) or so severely hypoxic, that their oxidative phosphorylation is severely reduced, while other tissues receive sufficient O <sub>2</sub> for oxidative phosphorylation.
COX	Cytochrome c oxidase
Critical O <sub>2</sub> pressure (COP):	The O <sub>2</sub> partial pressure (either measured externally or at the respiratory site) below which O <sub>2</sub> uptake diminishes (Armstrong <i>et al.</i> 2009; Sasidharan <i>et al.</i> 2017)
Energy crisis	When energy production via oxidative phosphorylation falls below optimum metabolic requirements
Energy crisis proteins	Proteins specifically involved with an energy crisis, occurring in both intolerant and tolerant tissues
ETC	Electron transfer chain in mitochondria
Hb-NO (or Pgb-NO) cycle	A metabolic cycle with inputs of NADPH, ADP, P <sub>i</sub> and O <sub>2</sub> and outputs of NADP and ATP while scavenging of NO and O <sub>2</sub> .
Hypoxia	O <sub>2</sub> concentrations below normoxic but more particularly values resulting in at least some cells within the exposed tissue/or organ being brought below the critical oxygen pressure
Key energy crisis proteins	those proteins which are highly expressed only in tissues tolerant to an energy crisis, i.e. which make the difference between anoxia tolerant and intolerant tissues
Messenger	Carrying certain information leading to a change in state (Felle, 2001).
Phytoglobins (Pgb or PGB)	An improved term for non-symbiotic plant haemoglobins (Hill <i>et al.</i> 2016)
Q <sub>10</sub> of O <sub>2</sub> consumption	$(O_2 \text{ uptake at } t+10)/(O_2 \text{ uptake at } t)$ , with t for temperature
Q <sub>max</sub>	Maximum rate of O <sub>2</sub> consumption
Well-aerated tissue	Tissue/organ in which all cells receive sufficient O <sub>2</sub> for optimum oxidative phosphorylation

## References

- Armstrong W, Beckett PM (1987) Internal aeration and the development of stelar anoxia in submerged roots. A multishelled mathematical model combining axial diffusion of oxygen in the cortex with radial losses to the stele, the wall layers and the rhizosphere. *New Phytologist* **105**, 221–245.
- Armstrong W, Webb T, Darwent M, Beckett PM (2009) Measuring and interpreting respiratory critical oxygen pressures in roots. *Annals of Botany* **103**, 281–293. doi:10.1093/aob/mcn177
- Atwell BJ, Greenway H, Colmer TD (2015) Efficient use of energy in anoxia-tolerant plants with focus on germinating rice seedlings. *New Phytologist* **206**, 36–56.
- Berry LJ, Norris WE (1949) Studies on onion root respiration. I. Velocity of oxygen consumption in different segments of root at different temperatures as a function of partial pressure of oxygen. *Biochimica and Biophysica Acta* **3**, 593–606.
- Brotel A, Kaiser WM (1997) Nitrate reductase activation state in barley roots in relation to the energy and carbohydrate status. *Planta* **201**, 496–501.
- Chang WWP, Huang L, Shen M, Webster C, Burlingame AL, Roberts JKM (2000) Patterns of protein synthesis and tolerance of anoxia in root tips of maize seedlings acclimated to a low oxygen environment, and identifications of proteins by mass spectrometry. *Plant Physiology* **122**, 295–317.
- Drew MC (1997) Oxygen deficiency and root metabolism: Injury and acclimation under hypoxia and anoxia. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 223–250.
- Felle HH (2001) pH: signal and messenger in plant cells. *Plant Biology* **3**, 577–591. doi:10.1055/s-2001-19372
- Felle HH (2005) pH regulation in anoxic plants. *Annals of Botany* **96**, 519–532.
- Frenkel C, Erez A (1996) Induction of chilling tolerance in cucumber (*Cucumis sativus*) seedlings by endogenous and applied ethanol. *Physiologia Plantarum* **96**, 593–600.
- Gibbs DJ *et al.* (2011) Homeostatic response to hypoxia is regulated by the N-end rule pathway. *Nature* **479**, 415–418.
- Gibbs J, Greenway H (2003) Mechanisms of anoxia tolerance in plants. I Growth, survival and anaerobic catabolism. *Functional Plant Biology* **30**, 1–47.
- Graciet E, Wellmer F. (2010) The plant N-end rule pathway: structure and functions. *Trends in Plant Science* **15**, 447–453.
- Hill R, Hargrove M, Arredondo-Peter R (2016) Phytoglobin: a novel nomenclature for plant globins accepted by the globin community at the 2014 XVIII conference on oxygen-binding and sensing proteins. *F1000Research* **5**, 212. doi:10.12688/f1000research.8133.1
- Lee RB (1979) The release of nitrite from barley roots in response to metabolic inhibitors, uncoupling agents and anoxia. *Journal Experimental Botany* **114**, 119–133.
- Paul MV, Lyer S, Amerkrauser C, Lehmann I, van Dongen JT, Geigenberger P (2016) Oxygen sensing via the ethylene response transcription factor RAP 2.12 affects plant performance under both normoxia and hypoxia. *Plant Physiology* **172**, 141–153.
- Saglio PH, Drew MC, Pradet A (1988) Metabolic acclimation to anoxia induced by low (2–4 kPa partial pressure) oxygen pretreatment (hypoxia) in root tips of *Zea mays*. *Plant Physiology* **86**, 61–66.
- Sasidharan R, Bailey-Serres J, Ashikari M, Atwell BJ, Colmer TD, Fagerstedt K, Fukao T, Geigenberger P, Hebelstrup KH, Hill RD, *et al.* (2017) Community recommendations on terminology and procedures used in flooding and low oxygen stress research. *New Phytologist* **214**, 1403–1407. doi:10.1111/nph.14519
- Sztark F, Tueux O, Erny P, Dabadie P, Mazat JP (1994) Effects of bupivacaine on cellular oxygen consumption and adenine nucleotide metabolism. *Anaesthesia & Analgesia* **78**, 335–339.
- Sztark F, Payen JF, Piriou V, Rigoulet M, Ventura-Clapier R, Mazat JP, Leverve X, Janvier G (1999), Metabolisme energetique cellulaire: aspects physiologiques et pathologiques. *Annales Françaises d'Anesthésia de Réanimation* **18**, 261–269.

- Zhang Q, Greenway H (1995) Membrane transport in anoxic rice coleoptiles and storage tissues of beet root. *Australian Journal of Plant Physiology* **22**, 965–975.
- Zhang Q, Lauchli A, Greenway H (1992) Effects of anoxia on solute leakage from beetroot storage tissue. *Journal of Experimental Botany* **43**, 897–905.