Tansley review

Efficient use of energy in anoxia-tolerant plants with focus on germinating rice seedlings

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Summary

Anoxia tolerance in plants is distinguished by direction of the sparse supply of energy to processes crucial to cell maintenance and sometimes to growth, as in rice seedlings. In anoxic rice coleoptiles energy is used to synthesise proteins, take up K⁺, synthesise cell walls and lipids, and in cell maintenance. Maintenance of electrochemical H⁺ gradients across the tonoplast and plasma membrane is crucial for solute compartmentation and thus survival. These gradients sustain some H⁺-solute cotransport and regulate cytoplasmic pH. Pyrophosphate (PPi), the alternative energy donor to ATP, allows direction of energy to the vacuolar H⁺-PPiase, sustaining H⁺ gradients across the tonoplast. When energy production is critically low, operation of a biochemical pHstat allows H⁺-solute cotransport across plasma membranes to continue for at least for 18 h. In active (e.g. growing) cells, PPi produced during substantial polymer synthesis allows conversion of PPi to ATP by PPi-phosphofructokinase (PFK). In quiescent cells with little polymer synthesis and associated PPi formation, the PPi required by the vacuolar H⁺-PPiase and UDPG pyrophosphorylase involved in sucrose mobilisation via sucrose synthase might be produced by conversion of ATP to PPi through reversible glycolytic enzymes, presumably pyruvate orthophosphate dikinase. These hypotheses need testing with species characterised by contrasting anoxia tolerance.

I. Introduction

‘Luctor et emergo’ I struggle and emerge.’

Shield of Zeeland, a maritime province of the Netherlands

The principal theme of this review is the efficiency of energy utilisation in plants during the energy crisis inherent in anoxia. We claim that the distinction between anoxia-intolerant and -tolerant species resides in efficient utilisation of energy rather than in ATP production during glycolysis; ethanol production (and therefore by inference ATP synthesis) can be as high in some anoxia-intolerant cells/tissues as in -tolerant ones (Greenway & Gibbs, 2003). Compelling evidence comes from cell suspension cultures of tolerant rice (Oryza sativa) and intolerant soybean (Glycine max) grown with 55 mM exogenous sucrose. These had similar rates of ethanol formation (c. 1 μmol mg⁻¹ protein h⁻¹)
after transfer to anoxia, yet subsequently the soybean cultures started dying after 2 d, whereas the rice cultures survived for at least 26 d (Mohanty et al., 1993; Supporting Information Notes S1.1). Consistently, in both Arabidopsis and rice after transfer to anoxia, transcripts increased for genes related to carbohydrate catabolism linked to fermentation (Narsai et al., 2009). A similar conclusion comes from a comparison of seven species, including Arabidopsis and rice, during exposure to anoxia or flooding (Mustroph et al., 2014). These observations do not contradict the importance of energy production for anoxia tolerance; increased energy demand, or very low energy production due to sugar starvation, may cause severe cell damage even in anoxia-tolerant species (Sections III.4. subsection ‘Role of the biochemical...’, III.5 ‘Release of free fatty acids indicates irretrievable cell injury’). Furthermore, ATP production during low external O₂, based on ethanol formation alone, may be an underestimate for some tissues, because mitochondria isolated from maize and rice roots form some ATP from the haemoglobin-nitric oxide (Hb–NO) cycle (Stoimenova et al., 2007) (see Box 1 for definitions and abbreviations used in this review).

Rice is the principal species considered in this review because it germinates and produces a coleoptile that grows in the dark under anoxia, providing a good model to understand the underlying physiology of anoxia tolerance. Survival (vis-à-vis growth) of coleoptiles during anoxia is best explored using excised coleoptiles after a period of recovery from the trauma of excision (Colmer et al., 2001). Excised coleoptile tips enable precise control of substrate supply and measurements of ion fluxes and rates of ethanol formation, whereas in intact seedlings results for coleoptiles are confounded by seeds as a source of endogenous substrates and ions, and also of ethanol formation. Excised coleoptile tips remain healthy but scarcely grow during anoxia and are therefore a good system for testing survival in anoxia. Complementary studies on intact seedlings during the first 4–5 d in anoxia are informative about growth, whereas the energy balance of coleoptiles would also be improved because K⁺ and sucrose can be derived via the phloem from the seed (Sections IV.1., IV.6.). In anoxia-intolerant tissues there is negligible phloem transport: in a maize system consisting of the scutellum and the main seminal root, phloem translocation of [14C]-labelled 2-deoxy-D-glucose to the root tip was inhibited by anoxia by 97% (Saglio, 1985; anoxic shock). By contrast, in anoxically germinated rice seedlings, sucrose import from the seeds to the coleoptiles was 2.7 μmol g⁻¹ FW coleoptile h⁻¹ (Edwards et al., 2012), which was half the rate of that in aerated seedlings and 80% of this sucrose was catabolised to ethanol. The response by germinating seeds and coleoptile growth by rice in anoxic soils differs to the situation in older plants which have access to O₂, either via emergent parts of the shoots above the floodwater or from photosynthesis when still submerged but above the soil (Colmer et al., 2014; Kirk et al., 2014).

During an energy crisis, pyrophosphate (PPi)-dependent enzymes such as the PP₁-consuming H⁺-translocase (V−H⁺PP,ase) and uridine diphosphate-glucose (UDPG) pyrophosphorylase involved in sucrose mobilisation via sucrose synthase (SuSy) are crucial to efficient energy flow to key processes essential to survival (Section II.3. subsection ‘Sucrose mobilisation...’: Fig. 1b,c).

Section III. discusses mechanisms of survival, analysing how specialised tissues and species can survive anoxia for 5 d or longer. Emphasis is on the maintenance of transmembrane H⁺ gradients which, in turn, maintain solute gradients and contribute to pH regulation, as well as on membrane structural integrity. Section IV. addresses the apportioning of energy in anoxia-tolerant rice seedlings on the basis of a recently constructed energy budget (modified from Edwards et al., 2012). The budget indicates that in these growing coleoptiles, energy expenditure during anoxia is mostly associated with a combination of protein turnover, accumulation of solutes (especially K⁺ and balancing anions) required for volume expansion, cell wall synthesis, lipid synthesis and cell maintenance. It is emphasised that the data on energy availability are inexact (Section IV.7.; Notes S3.1), but we consider them reasonable enough for current interpretation and to set the scene for further experiments.

II. PP₁-dependent enzymes as a key adaptation to an energy crisis, enabling survival and growth in anoxia

‘All animals are equal but some are more equal than others.’

Animal Farm, George Orwell (1945)

1. Introduction

PP₁-dependent enzymes are a key adaptation to anoxia, that is, during an energy crisis (Plaxton, 1996; Stitt, 1998; Gibbs & Greenway, 2003; Plaxton & Podesta, 2006; Plaxton & Tran, 2011). PP₁ has been aptly called an alternate energy donor (ED; Stitt, 1998; Plaxton & Podesta, 2006). These PP₁-dependent enzymes are central both to survival and limited growth under anoxia in the most anoxia-tolerant tissues/organs. In the coleoptile of germinating rice, these PP₁-dependent enzymes are very low in aerobic conditions, but greatly increase during anoxia (Section II.4.; Notes S2.1). PP₁-dependent enzymes also play a role in aerated tissues under some conditions, particularly during P₁ deficiency (Plaxton & Tran, 2011). In plants, the PP₁, formed by synthesis of polymers is often not hydrolysed (PP₁ → 2 P) via PP₁ases in the cytoplasm, as occurs in animals, instead entering a PP₁ pool in the cytosol (Ferjani et al., 2014; Fig. 1b,c). PP₁ concentrations in the cytosol in different plant tissues ranges between 0.2 mM (Maeshima, 2000) and 0.5 mM (Dancer et al., 1990; Plaxton & Tran, 2011). Within a particular tissue the PP₁ concentration is not readily changed by environmental conditions, such as P₁ deficiency, anoxia and metabolic poisons (Plaxton & Podesta, 2006). This stability of the PP₁ pool contrasts sharply with decreases in both ATP and the total adenylate pool in both the coleoptiles and roots of rice seedlings upon transfer to anoxia (Ricard & Pradet, 1989). Examples of similar PP₁ concentrations in anoxia and air are cultured cells of rice and soybean (Mohanty et al., 1993) and rice coleoptiles (Kato-Noguchi, 2002). This steady concentration of the PP₁ pool is consistent with the view that the PP₁-dependent enzymes act as a PP₁stat (Stitt, 1998; Notes S2.2). The PP₁ then supplies various crucial PP₁-consuming reactions such as UDPG pyrophosphorylase during sucrose mobilisation via SuSy, the
V-H$^{+}$PP$_{i}$ase and glycolytic PP$_{i}$-dependent reversible enzymes (Plaxton, 1996; Stitt, 1998; Maeshima, 2000; Huang et al., 2008). In principle, PP$_{i}$-dependent enzymes allow exchange between ATP and PP$_{i}$, aptly called ‘energy currencies’ (Huang et al., 2008). Hence, we refer in this review to the sum of NTP and PP$_{i}$ as ED; although they are not equivalent in the free energy of hydrolysis, being 43 kJ mol$^{-1}$ for ATP and 27 kJ mol$^{-1}$ for PP$_{i}$ (Davies et al., 1993, pH 7.3).

Overall, PP$_{i}$-dependent enzymes endow plants with a greater metabolic flexibility than animal cells (Plaxton, 1996; Stitt, 1998). Engagement of reversible PP$_{i}$-dependent enzymes during an energy crisis is often considered to increase the net ATP yield of glycolysis (Plaxton & Podesta, 2006; Huang et al., 2008; Igamberdiev & Kleczkowski, 2011). Equally importantly, scarce energy can preferentially flow to crucial processes catalysed by PP$_{i}$-dependent enzymes such as H$^{+}$ transport at the tonoplast (i.e. V-H$^{+}$PP$_{i}$ase, 

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**Box 1 Definitions and abbreviations used in this review**

**Definitions**

Acid load: undissociated organic acids which will permeate into the cytoplasm, through either the plasma membrane or tonoplast, and at the pH$_{cyt}$ will dissociate, producing H$^{+}$.

Active cells: cells which either are growing, or are in the acclimation phase following the onset of anoxia. Both types of cells have high metabolic activity.

Cell maintenance: maintaining the metabolic machinery of the cell, including proteins and membranes.

Donnan-free space: region outside plasma membrane, having a negative electrical potential of c. 20 mV.

Energy crisis: a substantial reduction in ATP synthesis occurring in anoxia, but also when oxidative phosphorylation is substantially reduced in air by other causes.

Flux equilibrium: when solute influxes are equal to solute effluxes.

Membrane integrity: when membranes have retained their structure and function.

Pasteur effect: acceleration of glycolysis, occurring in many species, particularly during early periods of anoxia.

pHstat biochemical: biochemical reactions which either dispose of H$^{+}$ by neutralising organic acids, or dispose of OH$^{-}$ by neutralising organic cations (Raven, 1986).

pHstat biophysical: in the present context, extrusion of H$^{+}$ across the plasma membrane.

Quiescent cells: nongrowing cells, which have acclimated to anoxia, hence metabolism needs only to ensure survival and thus can be relatively low.

Seed: used here as the term for caryopsis or grain. Following germination, it refers to the grain apart from the embryo (and growing seedling).

Turion: specialised overwintering bud in some aquatic plants.

**Abbreviations**

ADH: alcohol dehydrogenase.

ATP-PFK: ATP-dependent phosphofructokinase.

ED: energy donors (nucleotide triphosphates and pyrophosphate).

GDH: glutamate dehydrogenase.

Hb–NO cycle: a cycle involving NO$_{2}^{-}$ and NO$_{3}^{-}$, which includes haemoglobin, and produces some ATP during an energy crisis. The cycle requires only very low O$_{2}$ concentrations (Stoimenova et al., 2007).

K$^{+}_{cyt}$: K$^{+}$ concentration in cytoplasm.

K$^{+}_{in}$: concentration of substrate at which half of maximum velocity is attained.

NTP: nucleotide triphosphate (e.g. ATP, UTP).

OA: organic acid.

OA$^{-}$: organic anion.

PCD: programmed cell death.

PDC: pyruvate decarboxylase.

PEP: phosphoenolpyruvate.

pH$_{cyt}$ and pH$_{vac}$: pH of the cytoplasm and of the vacuole.

pH$_{ext}$: pH of the external medium.

PM-H$^{+}$ATPase: the ATP-consuming H$^{+}$-translocase at the plasma membrane.

PP$_{i}$: pyrophosphate.

PP$_{i}$-PFK: PP$_{i}$-dependent phosphofructokinase.

(P)PDK: pyruvate orthophosphate dikinase.

ROS: reactive oxygen species.

SuSy: sucrose synthase.

UDPG: uridine diphosphate glucose.

V-H$^{+}$ATPase: the ATP-consuming H$^{+}$-translocase at the tonoplast (vacuolar membrane).

V-H$^{+}$PP$_{i}$ase: the PP$_{i}$-consuming H$^{+}$-translocase at the tonoplast.
Fig. 1 Key role of pyrophosphate (PPi)-dependent enzymes in anoxia tolerance. (a) Aerobic catabolism produces large amounts of ATP, in turn allowing for rapid synthesis of polymers. (b) In anoxia with appreciable, although reduced, polymer synthesis (e.g. in acclimating cells and/or growing cells), there is still substantial PPi formation. This PPi is preferentially directed to two crucial processes, foremost the V-H+PPiase (i.e. vacuolar H+-translocating pyrophosphatase) at the tonoplast to maintain transmembrane gradients and also to uridine diphosphate glucose (UDPG) pyrophosphorylase which in sequence with sucrose synthase (SuSy) allows sugar mobilisation to yield hexose phosphates (instead of unphosphorylated hexoses by the alternative enzyme, invertase). Any surplus of PPi can be used by the reversible PPi-dependent glycolytic enzymes in the forward direction, further enhancing ATP yield for the same glycolytic flux.

Formation of AMP and PPi, during protein and lipid synthesis is also shown. The reporphosphorylation of this AMP can be achieved by two processes. First, by the pyruvate orthophosphate dikinase (PPDK) forward direction (Reaction 6). That process may reporphosphorylate all the AMP formed during lipid and protein synthesis, while consuming PPi in equimolar amounts. However, the PPi will flow through the PPi pool, to which UDPG pyrophosphorylase and the V-H+PPiase are likely to have preferential use. Second, PPi may not be enough to achieve the reporphosphorylation of AMP via PPDK and so adenylate kinase may be needed to reporphosphorylate some of the AMP (Reaction 9). (c) In anoxic cells with quite low polymer synthesis such as acclimated and nongrowing (‘quiescent’) cells, PPi production from polymer synthesis would be much reduced and so may be inadequate to provide sufficient PPi for V-H+PPiase and the UDPG pyrophosphorylase during sucrose mobilisation via SuSy. Hence, substantial PPi may have to be formed by the reversible PPi-dependent enzymes in the backward direction. These reactions constitute the ‘phosphofructokinase (PFK) substrate cycle’ (Table 1, Cycle 1) and the ‘pyruvate-phosphoenolpyruvate (PEP) substrate cycle’ (Table 1, Cycle 2). Of these, the pyruvate–PEP cycle is the more likely (see Section II.4. subsection ‘PPi formation, by NTP → NDP + PPi, . . . ’), but in both cases in vivo evidence is lacking. Either cycle would convert ATP to PPi. List of enzymes: 1a, invertase; 1b, SuSy; 1c, UDPG pyrophosphorylase; 2a, fructokinase; 2b, conversion of glucose-1-phosphate (G1P) to fructose-6-phosphate (F6P), via phosphoglucomutase and glucose isomerase; 2c, nucleoside diphosphate kinase; 3a, ATP-dependent phosphofructokinase (ATP-PFK); 3b, PPi-dependent phosphofructokinase (PPi-PFK); 4, phosphorpyruvating reactions between fructose 1,6-diphosphate and PEP; 5, pyruvate kinase; 6, pyruvate orthophosphate dikinase; 7, pyruvate decarboxylase and alcohol dehydrogenase; 8a, V-H+ATPase at the tonoplast; 8b, V-H+PPiase at the tonoplast; 9, adenylate kinase; 10, pyruvate dehydrogenase. Eqs 1–3, 7 and 10 (Nelson & Cox, 2008); Eqs 8a,b (Rea & Poole, 1993), Eqs 4–6 and 9 (Igamberdiev & Kleczkowski, 2011). ETC., electron transport chain (see also Table 1).
Table 1  Equations of reactions in which pyrophosphate (PPi) is formed and consumed in plant cells

<table>
<thead>
<tr>
<th>Equation number</th>
<th>Type of process</th>
<th>Process-enzyme</th>
<th>Equation</th>
<th>Comments on change in energy donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>PPi-producing reactions involving NTP → NDP + PPi</td>
<td>Sucrose synthesis</td>
<td>hexose-6-phosphate + UDPG → sucrose-6-phosphate + PPi</td>
<td>+1PPi - 2NTP: $ED = -1$ (Nelson &amp; Cox, 2008)</td>
</tr>
<tr>
<td>1b</td>
<td>Sucrose synthesis</td>
<td>sucrose-6-phosphate synthase</td>
<td>sucrose-6-phosphate → sucrose $+ P_i$</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Carbohydrate polymerisation</td>
<td>Polymern</td>
<td>$P_i + NTP + \text{glucose-1-phosphate} + \text{AMP} + \text{PPi}$</td>
<td>$+1PPi - 2NTP: ED = -1$ (Nelson &amp; Cox, 2008)</td>
</tr>
<tr>
<td>3</td>
<td>PPi-producing reactions involving NTP → AMP + PPi</td>
<td>Lipid synthesis</td>
<td>Acetyl-CoA synthetase</td>
<td>$\text{Acetyl-CoA} + \text{ATP} → \text{fatty acyl CoA} + \text{AMP} + \text{PPi}$</td>
</tr>
<tr>
<td>4</td>
<td>Amino acid incorporation into protein</td>
<td>Amino acid activation by transfer RNA, but not the possible requirement for amino acid synthesis (Nelson &amp; Cox, 2008)</td>
<td>$\text{Amino acid} + \text{ATP} + 2\text{GTP} → \text{peptide bond} + 1\text{AMP} + 1\text{PPi} + 2\text{GDP} + 2\text{Pi}$</td>
<td>$-3\text{NTP} + 1\text{PPi} + 1\text{AMP}: ED = -3$</td>
</tr>
<tr>
<td>5</td>
<td>Reactions to phosphorylate AMP</td>
<td>Pyruvate dikinase in forward direction</td>
<td>$\text{PEP} + \text{PPi} + \text{AMP} → \text{pyruvate} + \text{ATP} + \text{Pi}$</td>
<td>Uses PPi + AMP instead of ADP by phosphoenolpyruvate (PEP) kinase (Igamberdiev &amp; Kleczkowski, 2011)</td>
</tr>
<tr>
<td>6</td>
<td>Reversible PPi-dependent reactions in the glycolytic direction, consuming PPi</td>
<td>Adenylate kinase</td>
<td>$\text{AMP} + \text{ATP} → 2\text{ADP}$</td>
<td></td>
</tr>
<tr>
<td>7a</td>
<td>Sucrose mobilisation</td>
<td>Sucrose synthase</td>
<td>$\text{sucrose} + \text{UDP} → \text{UDPG} + \text{fructose}$</td>
<td>$-1\text{PPi} + 2\text{NTP}: ED = +1$ (Igamberdiev &amp; Kleczkowski, 2011)</td>
</tr>
<tr>
<td>7b</td>
<td>H+ transport at tonoplast</td>
<td>UDPG pyrophosphorylase</td>
<td>$\text{UDPG} + \text{PPi} → \text{glucose P} + \text{UTP}$</td>
<td>$\text{PPi hydrolysis energises H+ transport from cytoplasm to vacuole}$</td>
</tr>
<tr>
<td>8</td>
<td>Substrate cycle 1; PFK substrate cycle</td>
<td>V-H+PPiase</td>
<td>$\text{PPi hydrolysis energises H+ transport from cytoplasm to vacuole}$</td>
<td>Outcome of cycle: $1\text{ATP} → 1\text{PPi}$ (Nelson &amp; Cox, 2008)</td>
</tr>
<tr>
<td>9a</td>
<td>ATP-PFK</td>
<td>ATP-dependent phosphofructokinase</td>
<td>fructose 6P + ATP → fructose 1,6 diP + ADP</td>
<td>(Nelson &amp; Cox, 2008)</td>
</tr>
<tr>
<td>9b</td>
<td>PPi-PFK</td>
<td>PPi-dependent phosphofructokinase</td>
<td>fructose 1,6-diP + Pi $→ \text{fructose 6-P} + \text{PPi}$</td>
<td>(Stitt, 1998)</td>
</tr>
<tr>
<td>10a</td>
<td>PEP to pyruvate</td>
<td>Pyruvate kinase</td>
<td>$\text{PEP} + \text{ADP} → \text{pyruvate} + \text{ATP}$</td>
<td>In conjunction with adenylate kinase (Reaction 6) cycle 2 exchanges 1 ATP for 1 PPi (Igamberdiev &amp; Kleczkowski, 2011)</td>
</tr>
<tr>
<td>10b</td>
<td>Pyruvate to PEP</td>
<td>Pyruvate dikinase</td>
<td>$\text{pyruvate} + \text{ATP} + \text{Pi} → \text{PEP} + \text{PPi}$</td>
<td></td>
</tr>
</tbody>
</table>

‘Energy donor’ (ED) is the sum of ATP and PPi. Synthetic equations are based on Nelson & Cox (2008) and Maeshima (2000) and sucrose synthase on Igamberdiev & Kleczkowski (2011). PPi production by polymer synthesis can exceed (P) or be less than requirements (R) of the pyrophosphate (PPi)-dependent H+ translocase (V-H+PPiase) and sucrose mobilisation via sucrose synthase and uridine diphosphate glucose (UDPG) pyrophosphorylase. If $P > R$, then PPi can be used by pyrophosphate-dependent phosphofructokinase (PPi-PFK). If $P < R$, the PPi can be produced by the reversible glycolytic enzymes, most likely in the pyruvate-PEP substrate cycle (Section II.; Fig. 1b,c). UDP, uridine diphosphate; GTP, guanosine-5’-triphosphate; GDP, guanosine-5-diphosphate.
Carystinos et al., 1995; Gibbs & Greenway, 2003) and sucrose mobilisation (i.e. UDPG pyrophosphorylase involved in sucrose mobilisation via SuSy).

We first discuss polymer syntheses involving PPi formation, then the two crucial PPi-consuming enzymes: UDPG pyrophosphorylase, using the uridine diphosphate (UDP)-glucose formed from sucrose by SuSy, which increases net energy yield by glycolysis during anoxia by at least 25% (Fig. 1b,c; Section II.3. subsection ‘Sucrose mobilisation…’) and the V-H+PPiase, which is crucial to maintain intracellular compartmentation (Sections II.3. subsection ‘H+-transporting…’, III.3.). The reversible PPi-dependent glycolytic enzymes are then discussed (Section II.4.); these enzymes can either consume PPi, or, under other conditions, possibly produce PPi, depending on whether PPi produced from synthetic reactions exceeds or fails to meet requirements of UDPG pyrophosphorylase and the V-H+PPiase.

2. PPi formation during polymer synthesis

Polymer synthesis produces most of the PPi in eukaryotes (Maeshima, 2000; Ferjani et al., 2014). These biosynthetic enzymes fall into two groups. First, synthesis of sucrose, ...
hemicelluloses and cellulose are energised by NTP → NDP + PPi, (Table 1, Eqs 1a,b, 2; Maeshima, 2000). Synthesis of other polymers (protein and lipids) is energised by ATP → AMP + PPi, (Table 1, Eqs 3, 4). AMP then has to be converted to ADP to restore the balance of the adenine nucleotide pool, costing one NTP less than traditionally considered when the PPi produced is used in the conversion of AMP, but there is no net flow into the PPi pool.

3. PPi-consuming reactions during an energy crisis

PPi metabolism is an important acclimation response during an energy crisis, as indicated by large increases in transcripts and activity of a few key PPi-dependent enzymes (Section II.4.). As well, there may be activation of PPi-dependent enzymes due to increased cytoplasmic Mg2+, released due to a decrease in adenine nucleotides, as well as due to a decreased pH of the cytoplasm (pH cyt) (Igamberdiev & Kleczkowski, 2011). The effect of the decrease in pH cyt in rice coleoptiles is probably small, because pH cyt decreases only by c. 0.25 units (Section III.4. subsection ‘Observations at…’; see Davies et al. (1993) for the pH response of V-H+PPiase). In contrast to pH cyt, Mg2+ activation is more likely to apply to the coleoptile of rice seedlings, as the sum of adenine nucleotides was transiently decreased by 60% during the first 4 h following anoxic shock (Ricard & Pradet, 1989) accordingly increasing the free Mg2+ concentration. Stimulation of the V-H+PPiase would be particularly important during acclimation, when the total catalytic activity of the PPi-dependent enzymes is still low (for the increase with time of the V-H+PPiase after transfer of rice seedlings to anoxia, see Carystinos et al., 1995). In this experiment, extractable V-H+PPiase maximum catalytic activity declines during the first 2 h after return to air, and during the initial return to air there could also be fine control of in vivo activity that might be achieved by reductions in free Mg2+ in the cytosol associated with the rapid increase in the adenine nucleotide pool.

Sucrose mobilisation via sucrose synthase and UDPG pyrophosphorylase Sucrose mobilisation via SuSy and UDPG pyrophosphorylase, which is PPi dependent (Table 1, Eeq 7a,b), will produce two hexose phosphates per PPi spent (Fig. 1b,c), thus increasing the net ED production per mol sucrose catabolised to ethanol by 25% and NTP yield by 50%, compared with sucrose mobilisation via invertase (Fig 1a). The best demonstration of the acclimative value of sucrose hydrolysis via SuSy in anoxia is seen by comparing a maize double mutant with 6% of normal SuSy activity with its wild-type – after 24 h anoxia, the viability of root tips was reduced from 77% (wild-type) to 11% in the mutant (Ricard et al., 1998; hypoxically pretreated plants). SuSy was synthesized during anoxic germination in rice, maize and to a lesser extent wheat, whereas the activity of invertase remained negligible, so nearly all sucrose would be catabolised via SuSy (Guglielminetti et al., 1997). In contrast to intact seedlings, excised tissues are usually supplied with glucose rather than sucrose, so ATP production is then 2 mol mol−1 of hexose catabolised to ethanol which is inferior in ATP production per unit hexose catabolised to that of the coleoptile of intact seedlings with sucrose supplied from the seed; sucrose catabolism would provide 3 mol ATP mol−1 hexose catabolised to ethanol (Section IV.6.; Notes S3.2).

H+‐transporting pyrophosphatase at the tonoplast (V-H+PPiase) During an energy crisis the V-H+PPiase energises H+ transport from cytosol to vacuole by hydrolysis of PPi, thus constituting a crucial component of energy-dependent solute compartmentation (Table 1, Eqn 8; Figs 1b,c, 2b,c). The theoretical argument that PPi could energise H+ transport across the tonoplast was made by REA & POOLE (1993) and tested in seminal investigations by Carystinos et al. (1995), who documented a large increase in V-H+PPiase activity under anoxia and during chilling in rice seedlings (Carystinos et al., 1995; chilling Notes S2.3). After 4 d anoxia, this increase resulted in a 9-fold higher activity of the V-H+PPiase than of the V-H+ATPase, even though the specific activity of the latter had also increased by c. 2-fold. Further in vivo support for the importance of the V-H+PPiase, comes from Acr pseudoplatanus cells: when two inhibitors of phosphatases (imidophosphate and fluoride) were applied separately to cells depleted of energy by KCN, PPi levels were restored, whereas ΔpH across the tonoplast decreased by c. 3-fold due to the inhibition of the V-H+PPiase (Macri et al., 1995). Moreover, transcripts of the V-H+PPiase and ADH increased in a similar pattern between 0 and 18 h after start of anoxia (Carystinos et al., 1995). In a microarray analysis, Lasanthi-Kudahettige et al. (2007) showed that a specific copy of V-H+PPiase (Os02g55890) was upregulated 35-fold in anoxic rice coleoptiles. Similarly, V-H+PPiase transcript was among 44 upregulated genes, including alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC), observed in anoxic compared to aerated turions of Potamogeton distinctus. However, in that investigation changes in enzyme activity of V-H+PPiases and PDC, a key enzyme of ethanol formation, were small (Harada et al., 2007).

Six genes encode V-H+PPiases in the O. sativa genome, whereas only one isoform (OVP3) responded to anoxia started at germination (Liu et al., 2010). Increased expression was 10–17 times larger in the most anoxia-tolerant variety than in the least tolerant variety (Liu et al., 2010). Furthermore, in anoxia V-H+PPiase protein amounts increased, whereas V-H+ATPase protein decreased concurrently (Liu et al., 2010). Because the intolerant cultivar used by Liu et al. (2010) became much more anoxia tolerant in exogenous glucose (Huang et al., 2003b), it is of interest whether sugar supply to this cultivar would also increase V-H+PPiase expression and activity, or whether induction of this transporter is independent of sugar supply. Further preliminary evidence for a role of the V-H+PPiase in anoxia tolerance comes from experiments that manipulated the V-H+PPiase in rice (Notes S2.4). Further evidence for the key role of V-H+PPiase during anoxia comes from expression of an AVP gene (‘A’ signifies Arabidopsis) in yeast cells; these cells have no endogenous V-H+PPiase, whereas
the transgene resulted in acidification of the intravascular spaces of vacuolar membrane vesicles (Kim et al., 1994). Finally, Brauer et al. (1997) presented evidence for the action of the V-H’PPase in root hairs of maize; these findings were reviewed in detail (Greenway & Gibbs, 2003). A key finding was that 0.1–5 μM bafilomycin A1 (an inhibitor of V-H+ATPase) increased pH of the vacuole (pHvac) from 5.2 to 6.7 in aerated, but not in anoxic cells, in which pHvac increased only ‘slightly’ (Brauer et al., 1997; pHvac measured with a fluorescent probe). This supports the function of the V-H’PPase during anoxia.

A crucial question in view of the preferential flow of energy to the V-H’PPase is whether during an energy crisis other membranes also contain PPi-dependent H+-translocases (Notes S2.5). These have been detected for endomembranes of aerated plant tissues (Ferjani et al., 2014), but it is not yet known whether their capacity increases during anoxia.

4. Direction of flux via reversible PPi-dependent glycolytic enzymes

The enzymes PPi-dependent phosphofructokinase (PPi-PFK) and PPDK (Fig. 1b, c; Table 1, Eqs 9b, 10b) can in principle use or form PPi. PPi-PFK is induced by anoxia in rice from low levels in air to activities exceeding ATP-dependent phosphofructokinase (ATP-PFK) by 3- to 6-fold (cultured rice cells, Mohanty et al., 1993; rice coleoptiles, Mertens et al., 1990; Gibbs et al., 2000). Additionally, anoxic rice coleoptiles increased 4-fold in fructose 2,6-bisphosphate concentrations (Mertens et al., 1990), sufficient to activate PPi-PFK. This activation occurs in both the forward and reverse directions. However, there is no firm clue whether in vivo there would be a change in the direction of the net flux through this near equilibrium reaction (Stitt, 1990, 1998). Further, PPDK in the cytosol increased 1.5- to 3-fold in activity in anoxic rice roots (Moons et al., 1998). Consistently, PPDK protein in anoxic rice coleoptiles increased 3- to 10-fold in relative abundance (Huang et al., 2005a). Furthermore, PPDK transcripts in rice coleoptiles increased 365-fold (Lasanthy-Kudahettige et al., 2007) and 100-fold (Narsai et al., 2009).

PPi formation, by NTP → NDP + PPi, can either be higher or lower than the PPi requirements of UDPG pyrophosphorylase and the V-H’PPase

With the reduced activity of synthetic enzymes during an energy crisis, the question arises whether the PPi production in anoxic cells is higher, or lower, than the PPi requirements by the crucial enzymes UDPG pyrophosphorylase and V-H’PPase. In this speculative discussion, only synthetic reactions which are energised by NTP → NDP + PPi, are included, not reactions catalysing ATP → AMP + PPi, which, as stated above, always require either PPi or ATP to phosphorylate AMP to ADP (Section II.2.; Table 1, Eqs 5, 6).

PPi production exceeds demand

When rates of PPi production by polymer synthesis (involving NTP → NDP + PPi) exceed rates of PPi consumption by enzymes such as V-H’PPase and sucrose mobilisation via SuSy and UDPG pyrophosphorylase (Fig. 1a, b), the ‘excess’ PPi can be substituted for ATP by the reversible glycolytic enzyme PPi-PFK operating in the forward direction, rather than ATP-PFK (Table 1, Eqs 9a, b). During P deficiency and low temperatures, it has been assumed that formation of PPi is large enough to sustain the maximum possible increase in ATP formation during glycolysis, by assuming that PPi formation is sufficient to use PPi-PFK, rather than ATP-PFK, as the entry port of glycolysis (Plaxton, 1996; Plaxton & Tran, 2011). Similarly, in growing rice coleoptiles in anoxia, PPi formation during polymer synthesis may exceed the requirements of V-H’PPase and UDPG pyrophosphorylase (Igamberdiev & Kleczkowski, 2011).

PPi production lower than demand

PPi consumption by the crucial enzymes UDPG pyrophosphorylase and V-H’PPase may exceed the rates of PPi formation in polymer synthesis. This situation is likely when there is little or no growth (Maeshima, 2000) and under ‘stress’ (Plaxton & Podesta, 2006). During anoxia in the quiescent state, the formation of PPi would be through turnover of proteins and lipids (Sections IV.2., IV.4.). As stated above, both of these reactions form equimolar amounts of PPi and AMP; phosphorylation of AMP to ADP requires one ED, which would be spent either during the action of PPDK, or by adenylate kinase (Fig. 1b, c, Reactions 5, 6; Section II.2.). Either way, the formation of AMP + PPi, does not provide energy for other reactions; this contrasts with reactions which are energised by NTP → NDP + PPi, where the PPi becomes available for UDPG pyrophosphorylase and V-H’PPase. In the specific case of anoxic rice coleoptiles in Edwards et al. (2012), cell wall synthesis, the main reaction giving a net PPi flow into the pool, was only sufficient to provide sucrose mobilisation with PPi (Table 2), making it likely that at least part of the activity of V-H’PPase would require PPi to be produced by either of the two reversible PPi-dependent glycolytic enzymes, PPi-PFK or PPDK, thereby exchanging ATP for PPi. The PKF substrate cycle (Table 1, cycle 1; Fig. 1b,c) was proposed by Greenway & Gibbs (2003) but is unlikely because PFK may often be close to its maximum catalytic capacity to sustain glycolysis and therefore be unable to sustain the PKF cycle (Huang et al., 2008). The alternative source of PPi, is the PEP–pyruvate substrate cycle (Table 1, cycle 2; Fig. 1c), which has been aptly summarised by Igamberdiev & Kleczkowski (2011) as: ADP → PPi + AMP. AMP formed in this cycle will need to be converted by adenylate kinase to ADP (Table 1, cycle 2) completing the exchange of ATP for PPi. This cycle is much more likely to act in vivo than the PKF substrate cycle, because pyruvate kinase transcripts are increased by 4- to 8-fold during anoxia (Lasanthy-Kudahettige et al., 2007). Furthermore, pyruvate kinase attain a very high maximum catalytic capacity, sufficient to catalyse the conversion of PEP to pyruvate for both the completion of glycolysis and the pyruvate–PEP substrate cycle (Huang et al., 2008; Igamberdiev & Kleczkowski, 2011). It should be noted that, as far as we are aware, there is as yet no in vivo evidence for either cycle.

PPi production by the reversible glycolytic enzymes is predicted to be required in anoxic tissues in three situations: first, when energy demand is high, for example, during exposure
to a combination of anoxia and high NaCl, which almost certainly requires extra energy for Na⁺ extrusion and compartmentation; second, when substrates are depleted and it is crucial to direct scarce energy to the V-H⁺PPₐse to maintain tonoplast H⁺-pumping and to succrose mobilisation via SuSy and UDPG pyrophosphorylase; and third to provide PPᵢ for PPᵢ-PFK to accelerate glycolysis, that is, when glycolysis would otherwise be rate limited by ATP-PFK (Huang et al., 2008; Igamberdiev & Kleczkowski, 2011). In this third case, there would be an increase in ATP synthesis per cell, but no increase in the efficiency of energy yield (i.e. EDs produced per mol of sucrose catabolised), that is, the well-known Pasteur effect would be mediated by PPᵢ-PFK. Evidence for a function of PPᵢ-PFK in accelerating glycolysis was not supported when PPᵢ-PFK was reduced by 95% during wound respiration and anoxia, in anoxia-intolerant potato tuber slices (Stitt, 1998). However, the situation may well be different in anoxia tolerant tissues.

5. Summing up

The importance of directing the scarce energy resources to PPᵢ-dependent enzymes during an energy crisis should not hide the equally important contribution to acclimation by the large reductions in the rate of processes requiring energy, usually in the form of ATP. In intact rice seedlings, these reductions were 3-fold for protein synthesis, 2-fold for lipid synthesis and 5-fold for solute uptake (Edwards et al., 2012).

III. Mechanisms of survival during an energy crisis: that is, plant quiescence during anoxia with no growth and acclimated cells in anoxia

A key feature of acclimation to the low energy production under anoxia is a large reduction in energy costs for cell maintenance, including reduced ion fluxes (Section III.2.). Furthermore, reactions crucial to preventing cell death need preferential access to the scarce energy available, foremost to maintain transmembrane gradients (Section III.3. subsection ‘H⁺-transporting . . .’) and membrane integrity (Section III.5.). This highlights the crucial role of the V-H⁺PPₐse. When the energy crisis worsens, a biochemical pHStat can maintain transmembrane H⁺ gradients in anoxic rice coleoptiles at least for a further 18 h (Section III.4. subsection ‘Role of the biochemical . . .’).

1. Energy requirements for maintenance

Energy metabolism for maintenance can be studied during quiescence (i.e. in anoxia after the cells have acclimated) using excised tips of rice coleoptiles, which only grow imperceptibly after excision, but survive at least 120 h in anoxia provided that exogenous sugars are available (Greenway et al., 2011). Energy expenditure for maintenance of such quiescent tissues (between 50 and 120 h of anoxia) was c. 50% of the energy produced during acclimation to anoxia (0–24 h anoxia), based on ethanol formation in the minimum glucose concentration required for survival (Huang et al., 2005b).

In a previous review, energy requirements for maintenance of three moderate to extremely anoxia-tolerant species ranged between 0.26 and 0.67 µmol ATP mg⁻¹ protein h⁻¹ (Greenway & Gibbs, 2003). In excised rice coleoptile tips, the maintenance requirement was 0.4 µmol ATP mg⁻¹ protein h⁻¹ (Huang et al., 2005b). Even lower energy requirements for cell maintenance of 0.14–0.17 µmol ATP mg⁻¹ protein h⁻¹ are estimated for the rad (reduced alcohol dehydrogenase) rice mutant, which only develops a very short coleoptile under anoxia and by inference expends its fermentative produced energy mainly on cell maintenance (Edwards et al., 2012; Notes S3.3).

2. Reduction in ion fluxes – a critical element of downregulation of maintenance requirements

Reduced ion fluxes contribute to the restraint on energy expenditure during an energy crisis, these reductions may be due to less frequent opening of channels (Hochachka, 1986; for animal cells). Hochachka (1986) termed the phenomenon ‘channel arrest’, a term applied to plant membranes by Greenway & Gibbs (2003). However, downregulation of a NO₃⁻–H⁺ symport also occurs (Greenway et al., 2011), so ‘channel arrest’ should be replaced by ‘down regulation of solute fluxes’.

Economies in energy expenditure through diminished ion transport should be quite significant, because the ion fluxes in aerated conditions cost up to 50% of the energy required for maintenance (summarised in Greenway & Gibbs, 2003). Potassium fluxes across the plasma membrane of excised rice coleoptiles were reduced by c. 17-fold over 4 h of anoxia (Colmer et al., 2001; measured with Rb⁺). Similarly, Rb⁺ uptake by elongating coleoptiles was reduced 5-fold by anoxia, whereas efflux was negligible in anoxia, compared with an efflux amounting to 25% of Rb⁺ influx when in aerated solution (Edwards et al., 2012). The inferred changes in K⁺ permeability of the plasma membrane might be induced by changes in membrane potential and pHₓₓ. There can also be changes in various channels mediated by a large range of messengers, as deduced from studies on barley imposing high NaCl and H₂O₂ (Shabala & Pottosin, 2014; Shabala et al., 2014). Messenger molecules which ameliorate K⁺ loss and increase during anoxia include: putrescine (Shabala & Pottosin, 2014) and GABA (Shabala et al., 2014), as well as serine (Cuin & Shabala, 2007). Increases of these compounds in rice coleoptiles under anoxia were reported for putrescine (Reggiani et al., 1989), GABA (Ratcliffe, 1997) and serine (Greenway et al., 2011).

The net fluxes described above were measured over several hours, during which changes in the expression of genes encoding transporters could be expected. Thus, new experiments at high temporal resolution using microelectrodes are required (see Pang et al., 2006).

Potassium permeability was measured in tips of intact roots of flood-tolerant Vitus riparia while in anaerobic medium and forming ethanol at high rates (Manusco & Marras, 2006), even though shoots were in air so that strict anoxia was unlikely (Notes S2.6, S5). A 10-fold reduction in K⁺ influx during O₂ deprivation coincided with 7- to 8-fold reductions in K⁺ permeability, assessed from membrane depolarisations when external K⁺ was changed.
Table 2. Energy budget of anoxic rice coleoptiles describing use of nucleotide triphosphate (NTP), formation of pyrophosphate (PPi) and cost in energy donors (ED) based on rates measured by Edwards et al. (2012).

<table>
<thead>
<tr>
<th>Process (µmol g⁻¹ FW h⁻¹)</th>
<th>NTP used</th>
<th>PPi produced</th>
<th>Consumption in ED</th>
<th>Cost in ED (% of mean ED produced)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein synthesis (1.9)</td>
<td>36</td>
<td>+1</td>
<td>3</td>
<td>7.6 (37%)</td>
</tr>
<tr>
<td>Cell wall net synthesis (1.8)</td>
<td>2</td>
<td>+1</td>
<td>1</td>
<td>1.8 (9%)</td>
</tr>
<tr>
<td>Lipid synthesis (1.4)</td>
<td>2.8</td>
<td>+1</td>
<td>1</td>
<td>2.8 (14%)</td>
</tr>
<tr>
<td>K⁺ uptake from endosperm⁴ (0.9)</td>
<td></td>
<td></td>
<td></td>
<td>0–1.8</td>
</tr>
<tr>
<td>K⁺ uptake from medium⁵ (0.9)</td>
<td></td>
<td></td>
<td></td>
<td>0.9–2.7</td>
</tr>
<tr>
<td>Total K⁺ uptake (1.8)</td>
<td></td>
<td></td>
<td></td>
<td>0.9–4.5 (4.5–22%)</td>
</tr>
<tr>
<td>Sucrose transport</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Alanine synthesis (1.3)</td>
<td></td>
<td></td>
<td></td>
<td>0–1.3 (0–6.3%)</td>
</tr>
<tr>
<td>Sucrose mobilisation via sucrose synthase and UDPG pyrophosphorylase (2.2)</td>
<td></td>
<td>−1 PPi</td>
<td></td>
<td>2.2 (10.8%)</td>
</tr>
<tr>
<td>Total of presently measured processes: ATP produced during sugar catabolism¹</td>
<td></td>
<td></td>
<td></td>
<td>15.5–21.3</td>
</tr>
</tbody>
</table>

¹ Possible ratios of ATP/endproduct of glycolysis: 1.25, 1.5 and 2.0. Because the available efficiencies of ATP yield from fermentation give close to the same values for PPi production (in cell wall synthesis) and PPi, requirements of sucrose synthase we have taken the value 1.5 (Section II.3. subsection ‘Succrose mobilization. . .’; Supporting Information Notes S3.1); ²% is expressed on mean value for energy consumed.

For the present energy budget with elongating coleoptiles the maintenance costs cannot be assessed directly, but some of these maintenance requirements are included in various processes included in this table such as lipid and protein turnovers. Other energy costs for maintenance are unknown, for example, the costs to maintain cellular compartmentation. The rate of glycolysis in Edwards et al. (2012) is taken as the sum of ethanol, lipid and alanine synthesis. ED is NTP + PPi, and gives the total ATP consumption because PPi and ATP are exchangeable (see Section II.4.).
(Manusco & Marras, 2006). Simultaneous measurement of cell energy status, which changes rapidly upon transfer to anoxia (Mocquot et al., 1981), would also be valuable to detect the energy dependence of reduction of ion fluxes. To monitor influx and efflux across the tonoplast, longer-term experiments using radio-isotopes during flux equilibrium would be required (Luettge & Higinbotham, 1979; Notes S3.1).

3. Maintenance of transmembrane H+ electrochemical gradients by energised transport
As a central working hypothesis we have accepted the view of Felle (2005) that maintenance of transmembrane H+ gradients, which in plants provides the driving force for transport of solutes against free energy gradients, is crucial to survival during an energy crisis. Maintenance of transmembrane H+ gradients was indicated in anoxic rice coleoptiles where a range of exogenous solutes triggered an immediate membrane depolarisation, followed within minutes by partial repolarisation (Zhang & Greenway, 1995). This sequence indicates H+-solute cotransport, with longer-term disposal of H+ either by the PM-H+ATPase (this Section), or by the biochemical pHstat (Section III.4. subsection 'Role of the biochemical...'). These transmembrane gradients ensure maintenance of compartmentation and some energy-dependent solute transport, as well as keeping pHcyt at a ‘set point’, which in anoxia-tolerant tissues is only somewhat lower than that in air (Section III.4.). When transmembrane gradients fail due to an acute shortage of energy, compartmentation between vacuole and cytoplasm is lost, with fatal consequences. Thus the preferential direction of energy, via the V-H+PPase (Section II.3. subsection ‘H+-transporting...’; Fig. 1b,c) to maintain the H+ gradient across the tonoplast is indeed a crucial component of anoxia tolerance. In very anoxia-tolerant cells, modest H+-solute cotransport can be sustained by the residual activity of ATP-consuming H+-translocase at the plasma membrane (PM-H+ATPase) and by V-H+PPase activity (Fig. 2b). If energy provision deteriorates, or energy requirements increase, the PM-H+ATPase is unlikely to operate. During such an emergency, the energy required to sustain some H+-solute cotransport across the plasma membrane may be provided by a biochemical pHstat (Fig. 2c), very aptly termed a ‘battery’ (Mueller, 1968), because this pHstat was ‘loaded’ earlier when energy was plentiful. Because this pHstat is based on removal of organic acids, there will inevitably be loss of the counter ion K+ (Fig. 2c), otherwise there would be steep membrane depolarisation. When the ‘battery’ is exhausted compartmentation fails and death ensues (Fig. 2d; Felle, 2005).

4. pH regulation and the biochemical pHstat
**Observations at near-neutral exogenous pH** Upon exposure to anoxia, pHcyt in rice shoots and coleoptiles decreased to 7.15–7.2 over 24 h (Menegus et al., 1991; Kulichikhin et al., 2009), which is c. 0.3 units below the pHcyt of aerated tissues. As persuasively argued by Felle (2005), the decline in pHcyt within the first hour of anoxia is not a failure of regulation of pHcyt, rather it represents a new set point consistent with altered metabolism. Biochemical reactions critical to surviving a lower energy status are activated, whereas others are inhibited, depending on their pH optima (Felle, 2005). A good example of such regulation, although not involving anoxia, is that during Crassulacean acid metabolism; a drop in pHcyt of 0.3 units was sufficient to downregulate PEP carboxylase to allow a switch, from CO₂ dark fixation into malate at night to malate decarboxylation during the day (Hafke et al., 2001). In anoxic tissues, the decrease in pHcyt would reduce the free energy gradient across the plasma membrane and therefore reduce the energy cost of regulation (via H+ pumping) compared with a higher pHcyt (Raven, 2013). However, assuming an apoplastic pH of 5.0 (Felle, 2005), the reduction in energy costs of the maintenance of transmembrane gradients across the plasma membrane of the rice coleoptiles would only be c. 10%, because pHcyt in rice coleoptiles decreased only by 0.2–0.3 units (Menegus et al., 1991; Kulichikhin et al., 2009).

In contrast to the anoxia-tolerant organs of rice and Potamogeton, more distinct declines in pHcyt following exposure to anoxia occur in less anoxia-tolerant species, for example, in Medicago sativa root hairs from 7.3 to 6.8 (Felle, 1996) and from 7.45 to 7.05 in hypoxically pretreated maize root tips (Xia & Roberts, 1996). In maize root tips, the pHcyt of 7.05 declined further to pH 6.1 between 5 and 10 h. Such precipitous declines in pHcyt are unlikely to be the primary cause of anoxic injury, rather they may be due to a failing of transmembrane gradients and consequent loss of compartmentation (Felle, 2005).

In rice coleoptiles in a medium at pHext 6.5, the maintenance of the pHcyt, at 7.25–7.3 did not involve a substantial engagement of the biochemical pHstat (Greenway et al., 2011). Instead, PM-H+ATPase engagement in anoxic intact rice seedlings is indicated by net K+ uptake (c. 0.5 μmol g⁻¹ FW h⁻¹) from a medium containing 0.5 mM K+ (Huang et al., 2003a), uptake also sometimes occurring even in excised coleoptile tips (Greenway et al., 2011). This indicates some activity of the PM-H+ATPase over up to 5 d in anoxia, because uptake, or production, of the required balancing anion would tend to increase H+ in the cytoplasm, unless the PM-H+ATPase was engaged (i.e. Fig. 2b rather than 2c is operating). These observations support the view of Felle (2005) that surviving anoxia over long periods needs some residual H+ pumping to maintain transmembrane gradients.

**Role of the biochemical pHstat in maintaining transmembrane gradients as well as in pH regulation** The biochemical pHstat contributes to pH regulation and serves also as a ‘battery’ mechanism (energy supply) to maintain transmembrane gradients across the plasma membrane during an acute energy crisis. Evidence for a biochemical pHstat under anoxia includes: the formation of α-ketoglutarate and NO₃⁻ reduction (Ratcliffe, 1997; Felle, 2005) and malate decarboxylation (Roberts et al., 1992). Organic acids required for the pHstat would be mainly located in the vacuole, and hence their undissociated forms would constitute an acid load across the tonoplast (Fig. 2b). In rice coleoptiles, this acid load would be mitigated by the decrease in undissociated organic acids, the main species to permeate the tonoplast, due to an increase in pHvac from c. 5.3 to 5.9–6.1 in...
anoxia (Menegus et al., 1991; Kulichikhin et al., 2009); the upper limit of pH\textsubscript{vac} is c. 6.0 (Raven, 2013). This decrease in undissociated acid concentration reduces the cost of maintaining compartmentation. The increase in pH\textsubscript{vac} from c. 5 to 6 will also reduce the free energy gradient of H\textsuperscript{+} across the tonoplast and so the energy cost of H\textsuperscript{+} pumping from 11 to 7 kJ mol\textsuperscript{-1} (Notes S2.2).

For anoxic rice coleoptiles when in solution at pH of the external medium (pH\textsubscript{ext}) 6.5, there was little change in pH\textsubscript{cyt} and engagement of the pHstat was not required; of the organic acids, succinate even increased by 3 mM during 60 h anoxia (Greenway et al., 2011), presumably balanced by the small net K\textsuperscript{+} uptake of 0.1–0.2 μmol g\textsuperscript{-1} FW h\textsuperscript{-1} (Colmer et al., 2001; Kulichikhin et al., 2009; Greenway et al., 2011). Thus, there was no major change in the strong ion difference (Ullrich & Novacky, 1990; Notes S4) and no need for either substantial H\textsuperscript{+} extrusion or engagement of the biochemical pHstat. By contrast, when at pH\textsubscript{ext} 3.5 resulting in H\textsuperscript{+} entry, the provision of an emergency energy supply by a biochemical pHstat to maintain transmembrane gradients and pH regulation was evident, as indicated by decreases in malate and succinate over 18 h (Greenway et al., 2011; Fig. 2c). This pHstat presumably contributes to the remarkable tolerance of the rice coleoptiles to a combination of anoxia and pH 3.5. Consistent with the pHstat, K\textsuperscript{+} was lost continuously to the medium, in contrast to the small net influxes of K\textsuperscript{+} during anoxia at pH 6.5 (Fig. 2b,c). The pHstat requires transport of organic anion (OA\textsuperscript{−}), stored in the vacuole, through the tonoplast (Fig. 1c). This OA\textsuperscript{−} transport to the cytoplasm is almost certainly along a free energy gradient. Taking a potential difference of −20 mV across the tonoplast (towards the cytoplasm), for an anion there would be an uphill electrical gradient of c. 2 kJ, but that would easily be compensated for by a downhill concentration gradient of, for example, 6 kJ if OA\textsuperscript{−} concentration was 10 times higher in the vacuole than in the cytoplasm.

The effectiveness of a biochemical pHstat in rice coleoptiles for 18 h in anoxia, contrasts with aerated plant cells where it accommodates only short-term pH\textsubscript{cyt} fluctuations (Smith & Raven, 1979). Another difference between anoxia and air was that the provision of an emergency energy supply by a downhill concentration gradient of, for example, 6 kJ if OA\textsuperscript{−} concentration was 10 times higher in the vacuole than in the cytoplasm.

When there is engagement of the biochemical pHstat, however, the prolonged K\textsuperscript{+} losses (Fig. 2c) must eventually compromise survival, but causes for the eventual death in anoxia remain uncertain. We favour either complete collapse of transmembrane gradients and/or severe decline of membrane structure (i.e. loss of ‘integrity’). Alternatively, low K\textsuperscript{+} concentration in the cytoplasm could lead to diminished catalytic activity of key K\textsuperscript{+}-dependent enzymes and initiate programmed cell death (PCD) (Shabala et al., 2009; Demidchuk et al., 2010). This suggestion was based on data from animal systems, where a reduction in K\textsuperscript{+} from 100 to 50 mM in extracts and in cells resulted in a large increase in pro-caspase-3-like enzymes, which are involved in PCD (Hughes & Cidlowski, 1999). Shabala (2009) and Shabala & Pottosin (2014) and Shabala & Cuín (2007) extended this concept to plant cells, with the caveat that in plant cells K\textsubscript{cyt} may be kept high, even at low K\textsuperscript{+} concentrations in the cells as a whole. This K\textsuperscript{+} compartmentation was reviewed in detail by Leigh & Wyn-Jones (1986); in leaves of several species, the cytoplasm contained 90–120 mM K\textsuperscript{+}, even when vacuoles contained only 9–12 mM. Regarding rice, the excised coleoptiles held in anoxia for 120 h, without exogenous glucose, still contained c. 50 μmol g\textsuperscript{-1} FW, even though when held for between 3 and 5 d in anoxia these coleoptiles had ethanolic fermentation below the rate considered to be needed for maintenance (Huang et al., 2003a, 2005a). So, according to Leigh & Wyn-Jones (1986), the K\textsubscript{cyt} would still be 100 mM. Even so, these coleoptiles had much lower recovery of K\textsuperscript{+} and Cl\textsuperscript{−} uptake after return to aerated solution than glucose-supplied coleoptiles (Huang et al., 2005b), indicating injury. Possible explanations for the injury include the very low rate of ATP formation (as deduced from ethanol formation) could have led to injury due to other causes than low K\textsubscript{cyt}, for example, a deterioration of membrane integrity (Section III.5.). However, two other explanations, involving the notion that death is induced by low K\textsubscript{cyt} are: a portion of the cells in the coleoptiles may have K\textsubscript{cyt} lower than c. 50 μmol g\textsuperscript{-1} FW; and/or energy was insufficient to maintain high K\textsubscript{cyt}, that is compartmentation was failing due to inadequate energy formation (Fig. 1d). In rice coleoptiles, any adverse effect of low K\textsubscript{cyt} may be aggravated by lower activity of the V-H\textsuperscript{+}PP\textsubscript{ase}, which requires K\textsuperscript{+} at 60 mM for V\textsubscript{max} and has a K\textsubscript{m} for K\textsuperscript{+} of 11 mM (Obermeyer et al., 1996; Fig. 2d). Measurements of K\textsubscript{cyt} are needed to resolve this question.

A large vascular pool of K\textsuperscript{+} and balancing organic anions may be one reason for the long period that the highly vacuolated rice coleoptile can survive anoxia, while depending on energy from the ‘battery’ (Fig. 2c). The reason would be that large cells have a large volume : surface ratio and so the capacitance of the ‘battery’ would be large relative to the potential fluxes through the membranes. An additional contributing, or alternative, factor to the longevity of highly vacuolated cells may be the larger amounts of sugars for glycolysis in the vacuoles per unit cytoplasm than in slightly vacuolated cells. The magnitude of this effect can be gauged from data on pea roots; in highly vacuolated cells the volume ratio of vacuole : cytoplasm is c. 9 : 1, but in slightly vacuolated root tips only 0.66 : 1 (Spickett et al., 1993). This difference in capacitance of the biochemical pHstat, and in sugars stored to sustain catabolism, provides a rationale for the much lower anoxia tolerance in slightly vacuolated cells of maize roots than in highly vacuolated cells (Andrews et al., 1994), even though these tissues have similar levels of ADH per unit soluble protein (Andrews et al., 1994) and so presumably similar fermentation rates on a protein basis. Note that the ‘battery’, based on a net efflux of K\textsuperscript{+} (Fig. 2c), cannot work in growing cells because these require substantial K\textsuperscript{+} uptake as an osmolyte to sustain turgor. Accordingly, coleoptiles of intact seedlings that were exposed since excision to anoxia for 120 h, contrasts with aerated plant cells where it...
Summing up, under anoxia a biochemical pHstat provides not only pH regulation, but also acts as a ‘battery’ for an emergency supply of energy to maintain transmembrane gradients, both leading to loss of the balancing cation K\(^+\), which together with the organic anion had previously accumulated during periods of high energy supply.

5. Loss of membrane integrity

Supplementing the review by Greenway & Gibbs (2003), the present review concentrates on the question of whether loss of membrane integrity is associated with a lack of energy to maintain and renew membranes, or merely a secondary consequence of other metabolic perturbations. Lipid turnover uses considerable energy (Section IV.4.), so acute energy deficits would presumably compromise the integrity of membranes (Felle, 2005).

Electrolyte leakage to the bathing medium does not prove irretrievable injury, but loss of tonoplast integrity does Membrane integrity was maintained for at least 5 d in excised anoxic rice coleoptiles exposed to anoxia when in a solution at pH 6.5, as shown by retention of organic and inorganic solutes (Greenway et al., 2011). However, integrity might be jeopardised when substrates for glycolysis are depleted, as indicated by solute losses and poor recovery of sugar-starved coleoptiles after return to air (Huang et al., 2005b) and also of anoxia-tolerant beetroot tissues (Zhang et al., 1992). This lethal course of events is relevant to rice establishment in some flooded fields, when coleoptiles fail to reach light and carbohydrate supply is exhausted.

Solute losses are hard to interpret, because in tissues such losses may be associated with membrane depolarisations, transient deteriorations of many cells, or the release of solutes from a few individual cells which are dying. This question was resolved in red beetroot, where irretrievable injury was detected by loss of betacyanin, an endogenous marker of tonoplast integrity (Zhang et al., 1992). \(P_i\) losses have also been suggested as one of the best litmus tests for membrane injury for anoxic wheat shoots (Menegus et al., 1991). However, at least in rice coleoptiles, such \(P_i\) losses were only at times signs of irretrievable injury (Huang et al., 2005b).

Release of free fatty acids indicates irretrievable cell injury A promising indicator of irretrievable injury was the release, presumably from damaged membranes, of free fatty acids, detected in anoxic cultured potato cells, as reported in the seminal investigations by Rawyler et al. (1999, 2002). Free fatty acid release started at 10 h anoxia (Rawyler et al., 1999), which was accompanied by the appearance of dead cells (Pavelic et al., 2000). Nearly all membrane damage occurred during anoxia and may have been related to 2-fold decreases in ATP production between 6 and 12 h anoxia compared to 0–6 h (Rawyler et al., 1999, 2002; ATP formation was assessed from the end-products of glycolysis). Unfortunately, these key data cannot be compared with rice coleoptiles and other cultured cells (Section I.), because there are no data for protein concentrations, the only valid basis to compare species and tissues. These papers have provided valuable information on the processes of membrane deterioration, but the key question remains of whether the loss of membrane integrity is caused by insufficient energy for membrane turnover (Felle, 2005) or is the ultimate result of other lesions in metabolism. Such scenarios may well differ between anoxia-tolerant and -intolerant tissues. In the case of sugar-starved rice coleoptiles, a direct impact of energy shortage seems likely since addition of glucose during continued anoxia stimulated rice coleoptiles from 2 to 3 \(\mu\)mol g\(^{-1}\) FW h\(^{-1}\), whereas \(P_i\) loss for the following 24 h was reduced 9-fold (Colmer et al., 2001). For the potato cells, the primary cause of the membrane disintegration is much less certain, because the cells were in a high sucrose medium but no data on internal sugar concentrations were given.

Further investigation of both anoxia-tolerant and -intolerant tissues is required concerning the crucial issue of membrane stability (and thus integrity) during an energy crisis. For anoxic coleoptiles, data on lipid turnover are needed before and after provision of exogenous sugar while maintaining anoxia, together with more detailed patterns of changes in ethanol formation and solute loss after glucose addition. For anoxia-intolerant potato cells, the first test may be whether their endogenous sugar concentrations possibly run low, although it may also help to test recovery, for example, by uptake of Cl\(^-\) from low external concentration, which we suggest is an excellent litmus test for membrane integrity (Notes S1.2).

Membrane damage after return from anoxia to air is usually attributed to formation of reactive oxygen species (ROS; Blokhina et al., 2003). However, rice coleoptiles recovered rapidly after return to air (Huang et al., 2005b; Greenway et al., 2011) as did beetroot tissue (Zhang & Greenway, 1995). The best indications are repolarisations of beetroot cells within 10 min of return to air (Zhang & Greenway, 1995), which repolarised from \(-85\) to \(-165\) mV within 7 min at the end of a 72 h anoxia exposure. So, there was either little ROS formation, or there were effective systems for ROS disposal (Colmer et al., 2014). These systems were effective in rice leaves of 14-d-old plants: a genotype tolerant to complete submergence had 2.5 times less monodehydroascorbate, a lipid peroxide product, and 3–4 times more reduced ascorbate and activity of glutathione reductase than an intolerant genotype (Ella et al., 2003).

6. Summary

This section concentrates on mechanisms which enable quiescent cells to survive anoxia for up to 5 d. The key to survival is the maintenance of transmembrane gradients (Section III.3.), achieved by substantial reduction of ion fluxes (Section III.2.), the preferential direction of PP\(_i\) to V-H\(^+\)PP\(_{ase}\) (Section II.3. subsection ‘H\(^+\)-transporting…’ and probably some activity of the PM-H\(^+\)ATPase (Sections III.3., IV.1.), with resultant beneficial effects such as, in anoxic rice coleoptiles, regulation of pH\(_{cyt}\) within 0.15–0.2 units of aerated cells. When the demands for energy were increased by exposure to pH\(_{ext}\) 3.5, a biochemical pHstat became engaged, endowing survival of excised rice coleoptiles for at least another 18 h. In general, this pHstat functions both for pH regulation and as a ‘battery’ to maintain transmembrane gradients (Section III.4.}
subsection ‘Role of the biochemical. . .’; Fig. 2c), and would be engaged when either energy production became very low, or when energy demands increase such as happens with an acidic environment (e.g. pH \text{ext} of 3.5; Section III.4. subsection ‘Role of the biochemical. . .’), and we predict also when a combination of anoxia and salinity imposes demands on active Na\textsuperscript{+} transport.

Measured phospholipid compositions have so far given no clues as to how membranes are maintained (reviewed by Greenway & Gibbs, 2003), suggesting that a comparison of tissues in the following distinct phases is required: acclimatisation, quiescence, growth and necrosis. There are indications that irreversible membrane injury occurs (Fig. 2d) when energy becomes insufficient to renew the proteins and lipids of the membranes (Felle, 2005). That view is consistent with the rapid rates of turnover of lipids for growing coleoptiles, that would cost substantial energy (Section IV.4.).

The present section indicates it would be useful to obtain an energy budget for cells during quiescence, as discussed in the next section for elongating and acclimating cells.

IV. Energy expenditure during growth under anoxia

Rice coleoptiles and Potamogeton stems grow substantially during anoxia. Additional to the processes described in Section III. for quiescent cells, growing cells require: net solute uptake to sustain hydraulic gradients and thus turgor for volume expansion; net synthesis of cell wall polymers, proteins and possibly lipids; and net synthesis of glutamate needed in transaminations during synthesis of a range of amino acids, particularly of alanine (Section IV.5.). The energy costs of these three processes are described below, based on an energy budget (Table 2) for rice coleoptiles that increased 4-fold in length and 2-fold in FW during the fourth day after germinating in anoxic solution (Edwards et al., 2012). Overall, protein synthesis consumed c. 37\% of the energy and 14\% for lipid synthesis (Table 2). Substantial energy requirements can also be predicted when rice seedlings are transferred to anoxia after they have germinated in air; acclimation is achieved by a distinctive pattern of gene expression, with novel transcript profiles (Lasanthi-Perera et al., 2007) and ‘adaptive’ proteins (Huang et al., 2005a) becoming more abundant as cells respond to O\textsubscript{2} withdrawal (Narase et al., 2009; Shingaki-Wells et al., 2011).

In growing organs energy is spent both on growth processes and on cell maintenance. Maintenance is required, otherwise the cells will die. This section evaluates the apportioning of energy in anoxia-tolerant rice seedlings using data by Edwards et al. (2012). For the present energy budget with elongating coleoptiles the maintenance costs cannot be assessed directly, but some of these energy requirements are included in various processes measured by Edwards et al. (2012), such as lipid and protein turnovers of the tissues. Other energy costs for maintenance are unknown, for example, the cost to maintain the critical cellular compartmentation (Sections III.2., III.3.). The situation may be clarified by obtaining an energy budget for quiescent tissues, in which maintenance processes dominate. For nongrowing anoxic rice coleoptile tips the energy cost for maintenance has been assessed at 3.9 \textmu mol g\textsuperscript{−1} FW h\textsuperscript{−1} (Huang et al., 2005b); however, the possibility remains that in rapidly elongating coleoptiles maintenance costs may differ somewhat to these nongrowing tissues. So, Edwards et al. (2012) is an informative first formulation of an energy budget of an anoxia-tolerant tissue and provides a guide for further experimentation with a refined approach (Section V.).

1. Possible function of the plasma membrane H\textsuperscript{+}ATPase and transport of K\textsuperscript{+}

Tissues expanding under anoxia are likely to need some PM-H\textsuperscript{+}ATPase activity to enable solute uptake such as K\textsuperscript{+}, yet in most plant tissues exposed to anoxia, PM-H\textsuperscript{+}ATPase activity would be low or negligible (Felle, 2005). A well-proven exception is anoxic stems emerging from turions of P. distinctus, where in vivo activity of the PM-H\textsuperscript{+}ATPase was substantial, as shown by pronounced decreases in pH of the medium; this H\textsuperscript{+} pumping was accentuated by the stimulator fusicoccin and reduced by the inhibitor of PM-H\textsuperscript{+}ATPase, vanadate (Koizumi et al., 2010). Presumably, the PM-H\textsuperscript{+}ATPase supports the proton motive force required for K\textsuperscript{+} uptake (Koizumi et al., 2010), which must be associated with scavenging K\textsuperscript{+} which had leaked into the K\textsuperscript{+}-free medium. Indirect evidence for PM-H\textsuperscript{+}ATPase engagement in anoxic rice seedlings has been discussed in Sections III.3. and III.4. subsection ‘Observations at . . .’.

Expanding tissues need solutes to sustain the turgor pressure required for volume expansion, with K\textsuperscript{+} the dominant inorganic contributor (Avadhani et al., 1978; Atwell et al., 1982; Menegus et al., 1984). The source of K\textsuperscript{+} (exogenous vs unloaded from the phloem) affects the energetics of K\textsuperscript{+} acquisition. Potomageton stems elongate rapidly in deionised water (Summers et al., 2000; Koizumi et al., 2010), indicating solute translocation from turions to shoots. Such translocation occurred from seed to coleoptile of rice; the K\textsuperscript{+} content in the coleoptiles of rice seedlings in cultures without exogenous K\textsuperscript{+} increased by 20\% over 24 h (Huang et al., 2003a). Potassium uptake from the medium would require a substantial share of the scarce energy produced under anoxia, whereas high concentrations in the phloem derived from storage organs would supply K\textsuperscript{+} more economically. Interestingly, in anoxic rice roots the promoter of the anoxia inducible V-H\textsuperscript{+}PP\textsubscript{ase} (OVTP3) was only clearly expressed in the stele (Liu et al., 2010; Notes S2.5), which might contribute to the capacity for long-distance transport.

K\textsuperscript{+} derived from the internal reserves. The energy costs of K\textsuperscript{+} derived from the seed to the coleoptile are very uncertain. In cereal leaves, including rice, unloading of solutes from the phloem is via the symplast (Patrick, 2013), but the K\textsuperscript{+} flow into the coleoptile would be against a free energy gradient of c. 8 kJ. This value is based on: K\textsuperscript{+} concentrations in the phloem of 30–65 mM (Gould et al., 2004), whereas the electrical potential in sieve tubes could be as low as −155 mV (using values for willow from Wright & Fisher, 1981); K\textsuperscript{+} concentration in the cytosol of c. 100 mM (Section III.4. subsection ‘Role of the biochemical. . .’) and plasma membrane potential of −110 mV (Zhang et al., 1992). The costs of K\textsuperscript{+} transport from phloem to parenchyma cells of the coleoptile remain uncertain, primarily because unloading is through plasmodesmata and may be facilitated by mass flow (Gould et al., 2004).
Equally, there are uncertainties about the energy cost for K+ transport into the vacuole; the membrane potential of the tonoplast is \( c. \) 20 mV less negative on the vacuolar side (Niu et al., 1995), but although K+ movement would be against this electrical gradient, the K+ concentration in the cytosol would be expected to exceed that in the vacuole in anoxic rice coleoptiles (e.g. containing 50 \( \mu \text{mol} \) K+ g\(^{-1} \) FW, Huang et al., 2003a, 2005a) so the electro-chemical gradient could be favourable. Across the tonoplast, the concentration component of the free energy gradient for K+ would be about equal but in opposite direction to the electrical gradient (Section III.4. subsection ‘Role of the biochemical...’). So, it remains doubtful whether energy is needed for K+ transport from the cytosol into the vacuole, therefore the energy cost of endogenously derived K+ can only be given as 0–1.8 ED \( \mu \text{mol} \) g\(^{-1} \) FW h\(^{-1} \).

K+ taken up from the medium Potassium uptake from the medium would be more energy-expensive than translocation to the coleoptile from the seed. The energy cost associated with K+ uptake via a K+-H+ symport depends on ATP used per H+ extruded, which may be either two or one for the PM-H+\text{ATPase} (Warncke & Slayman, 1980). Even when the K+ uptake was along a free energy gradient via a channel, the energy requirement would arise from the extrusion of the H+ entering the cytosol either via anion cotransport, or produced during dissociation from organic acids, required to balance the K+. Additional energy required to transport K+ and the balancing anions across the tonoplast may amplify the total energy which at present can only be given as 0.9–2.7 \( \mu \text{mol} \) ED g\(^{-1} \) FW h\(^{-1} \) (Table 2).

Because growing coleoptiles in anoxia took up K+ from the medium and seeds in equal amounts (Edwards et al., 2012), the energy costs of K+ acquisition in this particular experiment would be 0.9–4.5 \( \mu \text{mol} \) ED g\(^{-1} \) FW h\(^{-1} \), being 4.5–22% of available energy (Table 2). However, this is for expending coleoptiles with solute demands, whereas ion fluxes would be much smaller in cells at, or near, flux equilibrium (Section III.2.; Box 1).

2. Protein synthesis

Net protein synthesis in anoxic rice coleoptiles can be as high as 50% of that in aerated coleoptiles (Alpi & Beever, 1983), yet in other experiments there was very little net protein synthesis (reviewed by Gibbs & Greenway, 2003). In the experiment by Edwards et al. (2012) that established the energy budget for anoxic rice coleoptiles, protein synthesis on an ethanol-insoluble DW basis was reduced by 88% in anoxia compared with aerated conditions; such a reduction in general protein synthesis, but with synthesis of specific proteins only, would save energy expenditure for those proteins needed for acclimation. Incorporation of amino acids into subcellular fractions of rice coleoptiles indicated substantial protein turnover in anoxia, even as the total protein pool remained steady (Atwell & ap Rees, 1986). The activity of individual ‘anaerobic’ proteins such as V-H\text{PPase}, which is critical to survival, increased linearly in anoxia (Carystinos et al., 1995). A deeper analysis of the dynamics of the proteome of individual subcellular compartments (e.g. Heazlewood et al., 2003) in anoxia-tolerant tissues is called for alongside time courses of gene expression (Lasanthisu-Kudahettige et al., 2007). A study in rice seedlings showed that a set of ‘core’ metabolites and transcripts characterised perturbations to O\(_2\) supply, with some increased and others decreased, in anoxia; these expressed genes presumably have a key role in survival during anoxia (Narsai et al., 2009).

Information on protein synthesis in anoxic rice coleoptiles comes from \(^{35}\text{S}\)-methionine incorporation into protein after 4 h labelling. The proportion of \(^{35}\text{S}\)-methionine incorporated as a proportion of methionine taken up was 70% in coleoptiles, 40% in embryos and only 25% in roots which did not grow under anoxia (Ricard & Pradet, 1989), emphasising preferential protein synthesis in the anoxic rice coleoptiles. Similarly, high rates of \(^{35}\text{S}\)-methionine incorporation were found in anoxic growing stems of \textit{Potamogeton} (Ishizawa et al., 1999; Dixon et al., 2006), arrowhead and rice coleoptiles (Ishizawa et al., 1999).

In excised tips of anoxic rice coleoptiles that had ceased to grow, the number of proteins synthesised (Fig. 1 in Huang et al., 2005a) is far higher than the 18 ‘anaerobic proteins’ identified in roots of maize, a less anoxia-tolerant species (Sachs et al., 1980, 1996). In 2-mm maize root tips, the number of proteins labelled during the first 4 h anoxia after a hypoxic pretreatment was higher than after anoxic shock, but again diminished to c. 20 proteins at 13 h anoxia, indicating that these maize root tips are inferior in protein synthesis under anoxia, compared to rice coleoptiles.

Significantly, in rice the number of labelled proteins was lower during quiescence than during acclimation; nine proteins highly labelled during acclimation (0–24 h anoxia) had decreased by 50% in activity after 72 h in anoxia (Huang et al., 2005a). Moreover, protein synthesis was much lower without exogenous glucose, coinciding with a lower rate of ethanol formation and by inference ATP formation (Huang et al., 2005a). This implied sensitivity of protein synthesis to energy availability is consistent with the fact that protein synthesis costs as much as 37% of available energy in anoxic coleoptiles, being 7.6 \( \mu \text{mol} \) ED g\(^{-1} \) FW h\(^{-1} \) (Table 2).

3. Cell wall synthesis

Cell walls of rice coleoptiles are largely celluloses and hemicelluloses (Zarra & Masuda, 1979), hence cell wall synthesis involves NTP → NDP + PP\(_i\) (Section II.2.). There is, as far as we know, little information on rates of cell wall synthesis in growing tissues under anoxia, with the only estimate for rice coleoptiles based on incorporation of exogenous [\(^{14}\text{C}\)]-labelled sucrose over 4 h of steady-state anoxia (Edwards et al., 2012), giving an estimate of hexose incorporation into cell walls of 0.5 \( \mu \text{mol} \) g\(^{-1} \) FW h\(^{-1} \). The endogenous sugar pools would not have been fully labelled, so rates would have been underestimated. Alternatively, cell wall synthesis can be estimated by subtracting protein mass from ethanol-insoluble DW. Estimates of the rate of cell wall synthesis give a rate which would cost \( c. \) 1.8 \( \mu \text{mol} \) ED g\(^{-1} \) FW h\(^{-1} \), that is, c. 9% of the energy consumed. On the one hand, this finding reinforces the view that the production of cellulosic cell walls required to make cells up to 300 \( \mu \text{m}\) long in submerged rice coleoptiles (Wada, 1961) is an inexpensive way of sustaining elongation. On the other, cell division is slow in anoxic rice coleoptiles, as cells are mainly present.
in the embryo, minimising the energy cost of synthesising proteins and replicating DNA in the coleoptile. For example, if protein had increased by the 40% over 24 h, in parallel with cell wall dry mass, the energy requirement would have been close to the entire ATP production during anoxia.

4. Lipid synthesis

The importance of lipid synthesis during anoxia in coleoptiles of rice seedlings is indicated by the incorporation of \([^{14}\text{C}]\)-sucrose into lipids over 4 h, with rates only 30% lower than in aerated tissues, compared to a 70–88% inhibition of protein synthesis during anoxia (Edwards et al., 2012). This is particularly remarkable as there was no net lipid synthesis between 3 and 4 d after germination (Edwards et al., 2012), confirming results in the classical paper of Vartapetian et al. (1978) who also recorded no net synthesis, but highlighted substantial lipid turnover during anoxia. Further, \([^{14}\text{C}]\)-acetate incorporation over 4 h, showed the label in anoxia compared to air had increased by 2.5-fold for neutral saturated lipids and decreased by 3-fold for phospholipids (Vartapetian et al., 1978). Generosova & Vartapetian (2005) suggested that the substantial turnover of phospholipids, glycolipids and fatty acids would function in stabilisation of membranes during anoxia. Also consistent are the prominent changes in genes for lipid synthesis in anaerobic germinating rice seedlings (Narsai et al., 2009). Regarding energy cost, each 2C incorporated into lipids of coleoptiles cost 1 ATP, and lipid synthesis cost 14% of the energy consumed, being 2.8 \(\mu\text{mol ED g}^{-1}\text{FW h}^{-1}\) (Table 2).

5. Amino acid synthesis

Here we only discuss alanine synthesis, which is the principal soluble amino acid accumulating under anoxia in many tissues (Gibbs & Greenway, 2003). In rapidly expanding rice coleoptiles, alanine was synthesised at \(c. 1.3 \mu\text{mol g}^{-1}\text{FW h}^{-1}\), being 0.18 times the amount of ethanol formation (calculated from Edwards et al., 2012), this synthesis sustained a quasi-steady alanine concentration of 30 \(\mu\text{mol g}^{-1}\text{FW}\), despite volume expansion. Similar rates of alanine synthesis were observed during the first 24 h anoxia in rice shoots (calculated from Menegus et al., 1984, 1993).

The endosperm supplies \(\leq 6\%\) of the alanine accumulated by rice shoots exposed to anoxia (Menegus et al., 1993). Instead, alanine synthesis consumes carbon skeletons from glycolysis (pea, Smith & ap Rees, 1979), whereas in rice seedlings the \(\text{NH}_4^+\) required for glutamate (the amino donor) is derived from storage amino acids imported from the seed (Menegus et al., 1993). Alanine formation would contribute substantially to ATP formation during steady-state growth and acclimation if the glutamate required for transamination was formed by glutamate dehydrogenase (GDH), which does not use ATP (Fig. 3). However, if glutamate were synthesised via glutamine, the cost would be 1 mol ATP mol\(^{-1}\) alanine (Fig. 3). Both reactions consume NADH, which would have been generated during glycolysis (Notes S3.4). Engagement of GDH in the direction of glutamate synthesis has been considered unlikely. Objectives to the possible engagement of GDH include the \(K_m\) for \(\text{NH}_4^+\) is 1 mM (Nelson & Cox, 2008; Santero et al., 2012), and \(\text{NH}_4^+\) in this range is usually considered to be inhibitory to metabolism (Taiz & Zeigler, 1991; Nelson & Cox, 2008). Reassuring evidence that glutamate synthesis by GDH may be feasible comes from Kronzucker et al. (1998), who assessed 20 mM \(\text{NH}_4^+\) in the cytoplasm of healthy rice roots. Presently, serious objections to glutamate formation by GDH remain: in excised rice coleoptiles of Calrose, a cultivar of moderate anoxia tolerance, labelling patterns with exogenous \(\text{NH}_4^+\) favoured the predominance of the enzyme sequence glutamine synthetase and glutamate synthase, rather than GDH (Gibbs, 1992); and alanine synthesis was inhibited when the alternative pathway glutamine synthase was inhibited in Medicago (Ricoul et al., 2005), a genus less anoxia tolerant than Oryza. Neither of these two investigations excludes the possibility of engagement of GDH in rice genotypes of very high anoxia tolerance. The diversion of pyruvate to lipids and amino acids (Sections IV.4., IV.5.) may or may not affect the rate of glycolysis and therefore the rate of ATP formation on a cell basis, depending on which factor is limiting the rate of glycolysis (Notes S3.5).

6. Sucrose catabolism and transport

Sucrose is the main substrate translocated in the phloem in most species, including rice (Patrick, 2013). Sugar is present in phloem sap at 200–600 mM (Patrick, 2013), compared with 10–60 mM in shoots of rice seedlings under severe hypoxia (stagnant solution) (Takahashi et al., 2014), which would mainly be coleoptiles, so sucrose unloading is along a large free energy gradient. Sucrose mobilisation via SuSy and UDPG pyrophosphorylase requires 1 mol PP\(_i\) mol\(^{-1}\) sucrose (Fig. 1b,c; Notes S3.2), so in our present budget using sucrose as a substrate costs 11% of assessed energy consumption (Table 2). However, this energy input provides a substantial return by producing 2 mol of hexose-phosphate mol\(^{-1}\) sucrose. Thus, for each hexose moiety derived from sucrose that is catabolised to ethanol, there is a net gain of 0.5 mol ATP, achieving because for the coleoptile of intact seedlings there is no cost of obtaining the sucrose; instead energy was spent in the seed on both the synthesis of sucrose from the glucose formed by the action of amylases on starch, and on the sucrose accumulation against a large free energy gradient into the phloem which then flows from the seed to coleoptile (Fig. 4).

7. Measurements of unidirectional fluxes of ATP synthesis are required for improving the energy budget

The energy budget of Edwards et al. (2012) has given insight into the energy expenditure of anoxic growing coleoptiles, but several imponderables remain. Regarding energy expenditure, there is at present no information on the energy required for RNA synthesis, which may be quite appreciable. More intrinsically, there appears to be no way in which possible costs for cell maintenance can be assessed directly for growing coleoptiles.

There is also uncertainty on the amount of ATP production in anoxic plant cells; calculations based on data by Edwards et al.
(2012) may well be an underestimate. We assumed a ratio of ATP-to-(end-product of glycolysis) of 1.5 mol mol$^{-1}$ (i.e. 3 mol ATP mol$^{-1}$ hexose catabolised) (see footnote in Table 2), whereas other authors assume 2 mol ATP mol$^{-1}$ end-product of glycolysis (i.e. 4 mol ATP mol$^{-1}$ hexose catabolised) (Huang et al., 2008; Igamberdiev & Kleczkowski, 2011; Edwards et al., 2012). An additional issue is that rates of RNA synthesis under anoxia are unknown; yet RNA synthesis would yield a net flow into the PP$\_i$ pool, because the NTP expenditure during RNA synthesis forms PP$\_i$ and NDP (i.e. similar to cellulose synthesis in Section II.2.). Importantly, energy supply might also be underestimated if there are end-products of sugar catabolism other than ethanol and...
alanine, as established in certain anoxic animal tissues (Hochachka & Lutz, 2001). However, the formation of ethanol accounted for the majority of carbohydrates catabolised in rice and tissues of plant storage organs (Smith & ap Rees, 1979). Another underestimate of ATP production would arise if ATP synthesis in the Hb–NO cycle (Stoimenova et al., 2007) occurs in vivo.

A major improvement in energy budgets would be measurements of unidirectional rates of ATP synthesis using in vivo saturation transfer \[^{31}\text{P}\]-NMR spectroscopy, which not only would give firm data on ATP production, but also indications as to whether the production exceeds the known expenditure. Any discrepancy would be valuable for further research and also indicate whether there might be substantial unaccounted maintenance costs. Unidirectional rates of ATP synthesis were measured in anoxic rice shoots, indicating that ATP formation was only 25% less in anoxia than in air (Fan et al., 1992). Gibbs & Greenway (2003) questioned the high estimate by Fan et al. (1992), partly because anoxia could not be assured, but also because it was 2–3 times higher than deduced from the rate of ethanol formation. Interestingly, another estimate of unidirectional ATP synthesis under anoxia gave only 6% of the rate in air, using anoxically shocked maize root tissues (Roberts et al., 1985). However, such a low value might be expected after anoxic shock (Notes S.1.1). Unidirectional rates would give the sum of ATP formation by sugar catabolism and any other sources (such as the possible Hb–NO cycle; Stoimenova et al., 2007) and by the exchange of PP, for ATP. Clearly, unidirectional rates of ATP synthesis would provide a major advance in elucidating anoxia tolerance. Such measurements should be conducted both for nongrowing quiescent (i.e. anoxia acclimated) tissues (the least complex situation), nongrowing tissues during the acclimation, and for growing tissues of rice coleoptiles and also other less tolerant tissues and species.

V. Conclusions on mechanisms of anoxia tolerance

This review centres on the coleoptile of rice and argues that in this anoxia-tolerant organ two energy donors ATP (and other NTP) and PP play a role during an energy crisis (Plaxton, 1996; Stitt, 1998), with PP, preferentially directing energy to reactions crucial to survival and growth such as the V-H’PPase and UDPG pyrophosphorylase involved in sucrose mobilisation via SuSy. Furthermore, we speculate that the flexibility endowed by PP-dependent enzymes of glycolysis includes their reversibility. The flux would be in the forward direction when synthetic reactions and therefore PP, production are substantial, substituting PP, for ATP-PFK during glycolysis and thereby increasing ATP yield per mol of hexose catabolised (Section II.4. subsection ‘PP, formation, by NTP → NDP + PP,→...’). However, during quiescence, PP, production by synthetic reactions may fall short of the requirements of the crucial enzymes V-H’PPase and UDPG pyrophosphorylase. In such cases, ATP could be exchanged for PP, via a reversible glycolytic enzyme, most likely PPDK rather than PP, (Section II.4.; Table 1, cycle 2 rather than cycle 1). This scenario is here proposed for anoxia-tolerant plant tissues, but to what extent this PP, metabolism is more dominant or even unique to anoxia-tolerant species requires evaluation. In a recent review, a comparison of seven species exposed to conditions leading to low O₂ or anoxia, highlighted *O. sativa* seedlings exposed to anoxia as the only case in which transcripts of all the four key PP-dependent enzymes (SuSy, PPi-PFK, PPDK and V-H’PPase) were increased (Mustroph et al., 2014). However, the type of low O₂ treatment varied in the different experiments (Notes S.2.6), so a true species comparison requires side-by-side experiments with anoxia as the treatment. Experiments to test the importance of PP, in both anoxia-tolerant and -intolerant species could use a transgenic approach with insertion of a soluble PP,ase (cf for hypoxic potato roots, Mustroph et al., 2005) and evaluations of the effects on metabolism and survival during anoxia.

In order to survive an energy crisis, transmembrane H⁺ gradients need to be retained, otherwise compartmentation of H⁺ ions and solutes will fail and death will ensue (Section III.3.; Fig. 2d). This retention of the membrane gradients is facilitated by a huge downregulation of ion fluxes; reducing the energy required for compartmentation between the vacuole and cytoplasm (Section III.3.) and allowing retention of H⁺-solute cotransport across the plasma membrane, albeit at greatly reduced rates (Section III.3.). The maintenance cost of compartmentation of ions and other solutes is at present unknown, because there have been no flux analysis and therefore no estimate of tonoplast fluxes under anoxia. Under an even more acute energy crisis, some H⁺-solute cotransport across the plasma membrane may be retained for c. 18 h by a biochemical pHstat, the H⁺ will combine with organic anions, whereas K⁺ previously balancing the organic anions will efflux across the plasma membrane, preventing substantial membrane depolarisation (Fig. 2c; Section III.4. subsection ‘Role of the biochemical...’). The second main feature of survival would be maintenance of membrane integrity (Section III.5.). As for the transmembrane H⁺ and ion gradients and fluxes, at the tonoplast, there are precious few data on this key aspect but it is likely that membrane deterioration is exacerbated by an acute energy shortage (Section III.5. subsection ‘Release of free fatty acids...’).

The energy budget for elongating coleoptiles of anoxic rice seedlings indicates that most energy was used for five processes: synthesis of proteins, solute uptake (and compartmentation) especially K⁺, cell wall synthesis, lipid synthesis and cell maintenance. The budget gives metabolic support for the long-held view that in anoxic coleoptiles, processes supporting elongation of preformed, small embryonic cells are critical for growth and thus survival and potential escape from anaerobic soil. Such conclusions highlight the need for an energy budget for nongrowing, acclimated cells. In the intact seedlings, the caryopsis expends energy to synthesise sucrose and its loading into the phloem, so improving the energy budget of the coleoptile (Sections II.3. subsection ‘Sucrose mobilisation…’, IV.6.). Present estimates of ATP synthesis remain in doubt because they are based on production of endproducts of glycolysis (Section IV.7.). A more direct approach is needed such as measurement of unidirectional ATP synthesis using in vivo \[^{31}\text{P}\]-NMR spectroscopy ATP saturation transfer (Section IV.7.). Confirmation of the hypotheses presented is required in a broader range of plant species and organs/tissues. An alluring possibility is that the preferential flow of energy, in the form of PP,
to reactions crucial to survival and growth, is characteristic of anoxia-tolerant tissues and less marked in anoxia-intolerant tissues. A principal aspect worth future attention is the suggested alleviation of the energy crisis in the coleoptile by the 'cheap' supply of sucrose and K⁺ from the seeds and whether these germinating seeds differ in this respect between anoxia-tolerant and -intolerant tissues (Sections IV.1., IV.6.; Fig. 4). In addition, it would be helpful to test the inferred higher anoxia tolerance of coleoptiles of intact seedlings compared with excised coleoptile tips, frequently used for their experimental convenience. Further research should prioritise the exploitation of genetic diversity within O. sativa and its progenitors by investigating genotypes with superior germination and growth rates in flooded soils (Ismail et al., 2011). Other useful species for experimentation on anoxia tolerance are the wild marsh plants, as has been shown for Potamogeton (Summers et al., 2000; Koizumi et al., 2010).

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Additional supporting information may be found in the online version of this article.

Notes S1 Techniques used to evaluate responses to anoxia.

Notes S2 Additional notes on PPi metabolism.

Notes S3 Additional notes on energetics and glycolysis.

Notes S4 Factors determining pH, the basis for using the strong ion difference (SID).

Notes S5 Problems with interpretation when roots are in an anoxic medium but shoots are in air.

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